Studies on the synthesis and delivery of biophosphates

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Studies on the Synthesis and Delivery of Biophosphates

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ABSTRACT

The synthesis of two inositol phosphates (part I) and a range of PEGylated polyamines (part II) is described. D-*myo*-Inositol 1,4,5-trisphosphate was prepared in five steps from *myo*-inositol in a synthesis involving a novel protection step - the incomplete hydrolysis of an orthoacetate orthoester to yield acetate protection. A sample was found to be equipotent for calcium release at the D-*myo*-Inositol 1,4,5-trisphosphate receptor compared to a commercial sample. DL-(1,3,5/2,4,6)-6-Hydroxymethylcyclohexane-1,2,3,4,5-pentol 1,2,4,7-tetrakisphosphate, a novel *scyllo*-inositol-based analogue of D-*myo*-inositol 1,3,4,5-tetrakisphosphate bearing a hydroxymethylphosphate at the *pseudo* 3-position, was synthesised from *myo*-inositol. It was found to be inactive at D-*myo*-inositol 1,4,5-trisphosphate receptors and was used as a standard in studies involving the biology of D-*myo*-inositol 1,4,5-trisphosphate 3-kinase. The polyanionic nature of biological phosphates such as inositol phosphates and DNA means it is difficult for these molecules to cross cell membranes; this is a particular problem for the delivery of DNA in gene therapy. For the second part of this thesis, attention is turned to the delivery of DNA. A range of polyamines conjugated to mPEGs were synthesised as potential gene delivery vectors. A number of compounds based on the linear tetramine thermine were constructed by coupling mPEG glycidyl ethers (of mPEG 550 and mPEG 2000) to Boc-protected thermine derivatives. Ethidium bromide assays suggested these conjugates formed complexes with DNA with weak interactions. A linear hexamine was synthesised by a cyanoethylation/reduction strategy from thermine and coupled to mPEG 550 again using the glycidyl ether. Two branched polyamines were synthesised, one pentamine and one triamine, and coupled to mPEG 550 *via* a spacer on their central nitrogen, using the mPEG 550 chloroformate. A stronger interaction with DNA was demonstrated for the hexamine based conjugates and the branched pentamine. The ability of three of the conjugates to transfer reporter genes to mouse muscle and tumour tissue *in vivo* was evaluated in tumour-bearing mice. Two of the conjugates were found to enhance reporter gene transfer to tumours compared to naked DNA.

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ABBREVIATIONS

Ac	acetyl
ADP	adenosine 5′-diphosphate
All	allyl
AMP	adenosine 5′-monophosphate
Ar	aryl
ATP	adenosine 5′ triphosphate
β-Gal	β-galactosidase
Bn	benzyl
Boc	*t*-butoxycarbonyl
Boc_2O	di-*t*-butyl dicarbonate
BOC-ON	2-(*t*-butoxycarbonyloxyimino)-2-phenylacetoniltrile
9-BBN	9-borabicyclo[3,3,1]nonane
bp	boiling point
Bu^t	*tert*-butyl
cADPR	cyclic adenosine diphosphate ribose
cAMP	adenosine cyclic 3′,5′-phosphate
camph	(–)-ω-camphanate
CAT	chloramphenicol acetyl transferase
Cbz	benzyloxycarbonyl
CLM	cytosol-like medium
COSY	correlation spectroscopy
CRAC	calcium-release activated channel
CTAP	N^{15}-cholesteryloxycarbonyl-3,7,12-tri-azapentadecane-1,15-diamine
DAG	1,2-di-*O*-acylglycerol
DC-Chol	3β-[N-(N',N'-dimethylaminoethyl) carbamoyl]cholesterol
DCC	N,N-dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DMRIE	N-(1,2-dimyristyloxypropyl)-dimethyl-(2-hydroxyethyl)ammonium bromide
DMSO	dimethylsulfoxide
DNA	deoxyribo nucleic acid
DOGS	dioctadecylamidoglycylspermine
DOPE	Dioleoyl-L-α-phosphatidylethanolamine
DOSPA	2,3-dioleyloxy-N-[2-(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate
DOTAP	1,2-dioleoyloxy-3-(trimethylammonio)propane
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
EC_{50}	concentration producing 50% of maximal response
EDTA	ethylenediaminetetraacetic acid
ES	electrospray
FAB	fast atom bombardment
Fmoc	9-fluorenylmethyloxycarbonyl
GAP	GTPase activator protein
GTP	guanosine 5′-triphosphate
GTPase	guanosine triphosphatase
h	hour
HBS	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hz	herz
IC_{50}	concentration causing 50% of ligand binding
Ins	inositol
Ins(1,4,5)	1D-*myo*-inositol 1,4,5-triphosphate
P_3	
IR	infrared
K_i	inhibition constant
LAH	lithium aluminium hydride
M	moles per litre

m/z	mass to charge ratio (mass spectrometry)
MALDI-TOF	matrix assisted laser desorption ionisation time of flight (mass spectrometry)
MCPBA	3-chloroperoxybenzoic acid
Me	methyl
MES	4-morpholineethansulfonic acid
MHz	megahertz
min	minute(s)
MIPP	multiple inositol polyphosphate phosphatase
mp	melting point
mPEG	poly(ethylene glycol) monomethyl ether
NCA	N-carboxy anhydride
NMR	nuclear magnetic resonance
PEG	poly(ethylene glycol)
PEI	polyethylenimine
Ph	phenyl
PH	plekstrin homology
Phth	phthalimido
PINC	protective interactive non-condensing
Piv	pivaloyl
PLC	phospholipase C
PLL	poly-L-lysine
PMB	4-methoxybenzyl
ppm	parts per million
Pr^i	*Iso*propyl
PtdIns	phosphatidylinositol
PTSA	4-toluenesulfonic acid
PVA	polyvinyl alcohol
PVP	polyvinylpyrrolidone
TFA	trifluoroacetic acid
TFAc	trifluoroacetyl
THF	tetrahydrofuran
TLC	thin-layer chromatography
TMS	tetramethyl silane
Troc	2,2,2-trichloroethyloxycarbonyl
Ts	tosyl
TSP	trimethylsilyl propionic acid

CONTENTS

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PART I:

SYNTHESIS OF INOSITOL POLYPHOSPHATES

The physiological significance of the stimulation by acetylcholine of the turnover of phosphate and phosphoryl units in the phospholipids of pancreas and brain remains unknown, although these and earlier studies do point to the phospholipids as participating in the physiological events thrown into play by acetylcholine.

[Hokin and Hokin, 1955]

Micromolar concentrations of inositol 1,4,5-trisphosphate release Ca^{2+} from a non-mitochondrial intracellular Ca^{2+} store in pancreatic acinar cells. Our results strongly suggest that this is the same Ca^{2+} store that is released by acetylcholine.

[Streb *et al*, 1983]

Chapter 1:

The Phosphoinositide System

1 Transmembrane signalling

Communication between cells in a multicellular organism is essential for all physiological processes. A range of substances, from small molecules to proteins mediate this intercellular communication. The majority of these substances (hormones, neurotransmitters, cytokines, growth factors, eicosanoids), so-called *first messengers*, are charged hydrophilic molecules and are unable to pass through the lipid bilayer of the plasma membrane. Therefore, in order to exert their effect on cells they stimulate extracellular receptors causing the release or production of a substance within the cell, a so called *second messenger*. It is the second messenger that elicits the cellular response. This arrangement also provides a means of amplifying the signal across the cell membrane, one molecule of extracellular agonist leads to the production of many molecules of second messenger. Further, the same agonist may elicit different responses in different tissues through its receptors utilising different second messengers. There are a number of second messengers. The first part of this thesis is concerned with the second messenger D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3‡ Figure **1**].

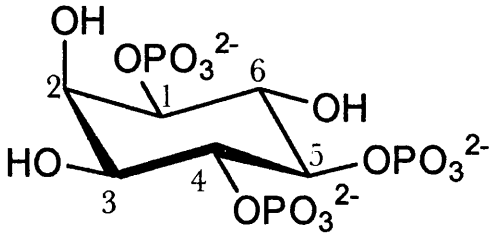


Figure 1 D-*myo*-inositol 1,4,5-trisphosphate

2 Calcium ions

One of the most versatile and universal signalling agents in the human body is the calcium ion.

[Berridge *et al*, 1998]

Calcium ions are used as an intracellular signal in many processes including muscle contraction, secretion, metabolism, neuronal excitability and proliferation. When intracellular Ca^{2+} concentration is low (10-100nM range) cells remain quiescent. With an increase in Ca^{2+} concentration (500-1000nM range), cells are activated to perform their particular functions *via* Ca^{2+} sensors such as calmodulin or troponin C, which transduce the signal into specific cellular responses. Therefore, the activity of a cell depends upon the balance between Ca^{2+} "ON" mechanisms and Ca^{2+} "OFF" mechanisms. Intracellular Ca^{2+} concentration may rise by Ca^{2+} entry from outside the cell or by release of Ca^{2+} from intracellular stores. Ca^{2+} concentration

‡ This style of abbreviation for inositol phosphates shall be used throughout. The numbers in brackets refer to the positions on the inositol ring of the phosphate groups, and compounds are D-*myo* stereoisomers unless indicated otherwise.

outside the cell is 10000 times greater than that inside and, as the inside is also negatively charged, there is a strong electrochemical gradient in favour of Ca^{2+} entry.

The plasma membrane may contain a number Ca^{2+} channels, including voltage-operated channels found mainly in excitable cells, which open in response to membrane depolarisation, receptor-operated channels activated by extracellular agonists and calcium-release activated channels (CRACs) activated by emptying of intracellular Ca^{2+} stores through a mechanism called capacitative Ca^{2+} entry. When the endoplasmic reticulum Ca^{2+} store is full, entry of Ca^{2+} through CRACs is inhibited but, when it discharges, the CRACs open. The mechanism by which this modulation occurs is not known. It has been suggested [Randriamampita and Tsien, 1993] that the empty Ca^{2+} stores release a messenger *calcium influx factor* that diffuses to the membrane and opens the CRACs.

Release of Ca^{2+} from intracellular stores occurs *via* two main types of Ca^{2+} channels; the $Ins(1,4,5)P_3$ receptor and the ryanodine receptor. Ca^{2+} release involving the $Ins(1,4,5)P_3$ receptor will be considered in detail later. Cyclic adenosine diphosphate-ribose (cADP-ribose, Figure **2**), a metabolite of nicotinamide adenine dinucleotide (NAD), is the endogenous Ca^{2+} releasing ligand for the ryanodine receptor; its main signalling function is modulation of Ca^{2+} induced Ca^{2+} release [Lee, 1997]. The metabolic signals that regulate cADP-ribose production and the termination of its action are unknown and our knowledge of its metabolic enzymes is limited. NAD is converted to cADP-ribose by ADPR cyclase and it is metabolised by cADP-ribose hydrolase, an enzyme that may have other functions. Recently, a third molecule capable of releasing Ca^{2+} from intracellular stores has been described, nicotinic acid adenine dinucleotide phosphate (NAADP) [Genazzani and Galione, 1997; Lee, 1997]. The release mechanism, which is not known, and the stores on which NAADP acts are distinct from those of cADP-ribose and $Ins(1,4,5)P_3$.

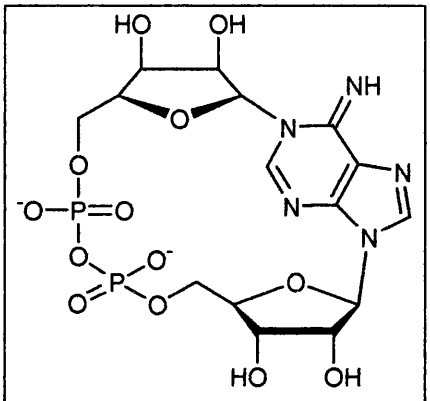


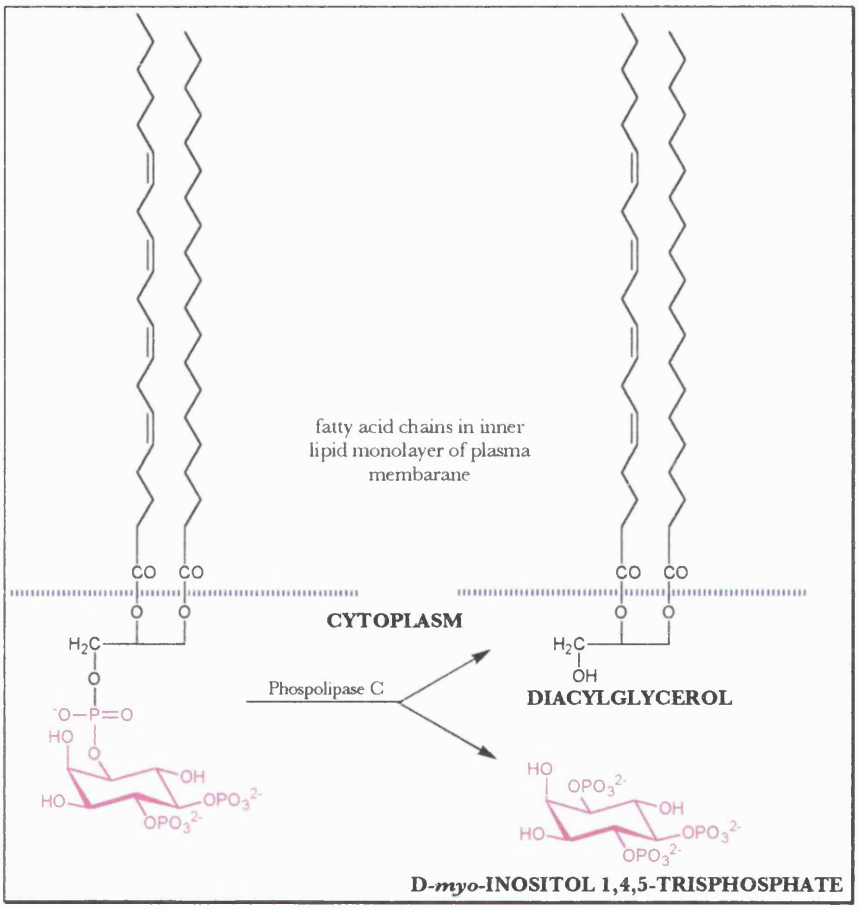
Figure 2 Cyclic adenosine diphosphate-ribose

Once intracellular Ca^{2+} concentration has risen and the cell has responded, the cell must return to its original state in order to receive further signals. There are a number of Ca^{2+} "OFF" mechanisms. Plasma membrane Ca^{2+} ATPases utilise the energy of ATP to extrude Ca^{2+} from the cell against the electrochemical gradient; in addition, excitable cells use a Na^+/Ca^{2+} exchanger system. Ca^{2+} ATPases also operate in the endoplasmic reticulum membrane to replenish intracellular stores.

3 Phosphoinositide signalling

3.1 Introduction

The membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is cleaved by phospholipase C (PLC) releasing Ins(1,4,5)P_3 into the cytosol (scheme **1**). The other product of this pathway is diacylglycerol (DAG). Both DAG and Ins(1,4,5)P_3 act as second messengers. Ins(1,4,5)P_3 stimulates Ca^{2+} release from intracellular stores, DAG activates protein kinase-C (PKC).



Scheme 1 Hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C.

3.2 Diacylglycerol

The cellular response to DAG is mediated *via* the activation of PKC[†] which phosphorylates serine and threonine residues in many target proteins and is implicated in a diverse range of cellular processes. The regulation of PKC is the subject of a recent review [Liu and Heckman, 1998]. Some PKC isozymes are activated by the tumour-promoting phorbol esters and are thus thought to affect some aspects of cell cycling. DAG is metabolised by two major pathways. In

[†] In fact, a family of 11 different PKC isozymes are now known, not all of which are activated by DAG [Liu and Heckman, 1998].

the first, phosphorylation by a kinase enzyme to phosphatidic acid is followed by combination with cytidine 5'-monophosphate (CMP) to form CMP-phosphatidate, this in turn is coupled to *myo*-inositol to form phosphatidyl inositol and ultimately PtdIns(4,5)P_2. In the second, hydrolysis by a lipase leads to the formation of monoacylglycerol and arachidonate, the latter of which is used for the synthesis of various eicosanoids.

3.3 Cellular Production of D-*myo*-Inositol 1,4,5-Trisphosphate

In 1983, Ins(1,4,5)P_3 was identified as the phospholipid derived mediator of increased intracellular Ca^{2+} levels [Streb *et al*, 1983]. The events that lead to Ins(1,4,5)P_3 production have subsequently been extensively studied and are now well established [Berridge, 1993]. Ins(1,4,5)P_3 is produced when an agonist binds to an extracellular receptor, causing activation of PLC. Many isoforms of PLC have been identified and have been classified into three main groups β, γ and δ. Activation of PLC occurs through two major types of receptor; those coupled to GTP-binding proteins (G-proteins) and those linked to tyrosine kinase. There are numerous G-protein coupled receptors and most have seven transmembrane domains. Agonist stimulation induces a conformational change in the receptor which causes the heterotrimeric G-protein to dissociate into G_α and $G_{\beta\gamma}$ subunits, both of which can activate different PLC isoforms. For example PLCβ1 is activated by the α subunit of the G-protein G_q. The tyrosine kinase-linked receptors relay information through a direct interaction between the receptor and PLCγ1. Upon agonist stimulation, the receptor dimerises and undergoes autophosphorylation of specific tyrosine residues. This provides a binding site for PLCγ1, which is then phosphorylated and begins to hydrolyse PtdIns(4,5)P_2. It is not known which receptors or transducers are coupled to PLCδ members [Rhee and Choi, 1992]. Some examples of receptors that are now known to utilise Ins(1,4,5)P_3 are listed in table **1**.

Via PLC-β	Via PLC-γ
Adrenoceptors: α_{1A} α_{1B} α_{1D}	Epidermal growth factor receptor
Angiotensin: AT_1	Fibroblast growth factor receptor
Bradykinin: BB_1 BB_2	Platelet-derived growth factor receptor
Cholecystokinin: CCK_A CCK_B	T cell antigen receptor
Histamine: H_1	
5-HT_{2A} 5-HT_{2B} 5-HT_{2C}	
Glutamate: $mGlu_1$ $mGlu_5$	
Muscarinic: M_1 M_3 M_5	
Thrombin	

Table 1 Examples of receptors coupled to PtdIns(4,5)P_2 turnover

3.4 D-*myo*-Inositol 1,4,5 Trisphosphate Receptors

The Ins(1,4,5)P_3 receptors are tetramers. Each subunit consists of three domains: an Ins(1,4,5)P_3 binding N-terminal domain, a regulatory domain containing ATP-binding and phosphorylation sites and a C-terminal domain containing six membrane-spanning regions. Both the N-terminal and regulatory domains lie free in the cytosol and the C-terminal regions co-operate to form the Ca^{2+} channel. The highly conserved N-terminal region incorporates some 650 amino acid residues, rich in positively charged arginine and lysine that may have a role in binding the phosphate groups. Upon binding Ins(1,4,5)P_3, the receptor undergoes a large conformational change leading to Ca^{2+} channel opening.

Three isoforms have so far been firmly identified, designated type I, II and III; all three have been cloned and sequenced. Based on rat sequences, type II has 69% identity with type I, type III has 62% [Joseph, 1996]. Fourth and fifth isoforms have been reported based on partial sequence information, both having a high degree of identity with type II. Studies using isoform-specific antibodies suggest that all cells contain multiple receptor isoforms but that one isoform may predominate in a particular tissue or cell type. For example, type I receptors are the major isoform found in the brain, particularly enriched in cerebellar Purkinje cells. Furthermore, a recent study indicates that these receptors are essential for proper brain function [Matsumoto *et al*, 1996]. In this study, mice lacking type I Ins(1,4,5)P_3 receptors were generated by gene targeting. Most of the mice died *in utero*; those that survived had severe ataxia and tonic or tonic-clonic seizures and died by the weaning period. Electroencephalography showed that the mice suffered from epilepsy. Gross examination of the brain found a reduced size but an apparently normal structure; the gross structure of various peripheral tissues appeared normal. Light-microscopic examination of the brain and various peripheral tissues showed no significant difference from normal mice.

Ins(1,4,5)P_3 receptors are largely located in the endoplasmic reticulum membrane as would be expected, although they have been shown to be present in the plasma membrane in a number of experimental systems [Joseph, 1996]. They have also been found in the outer nuclear membrane although, given the contiguity of this membrane with the endoplasmic reticulum membrane, this is not surprising.

3.5 Structure-Activity Relationships at Ins(1,4,5)P_3 Receptors

A large number Ins(1,4,5)P_3 analogues have now been synthesised and it has become obvious that the Ins(1,4,5)P_3 receptor shows considerable stereo- and regio-specificity in its structural requirements for binding and Ca^{2+} release. The stereospecificity of the receptor is clearly demonstrated by the fact that L-Ins(1,4,5)P_3 does not release Ca^{2+} and its binding is 2000 times

weaker than that of Ins(1,4,5)P_3. Further, this collection of analogues has allowed a picture to be built up of what substituents on the ring are conducive to binding and Ca^{2+} release [Potter and Lampe, 1995]. It is the 4,5-bisphosphate arrangement that is accepted as the essential pharmacophore; deletion of either the 4- or the 5-phosphate abolishes activity. Vicinal phosphates at other positions on an inositol ring can mimic this 4,5-bisphosphate arrangement to varying degrees. The 6-hydroxyl is important for binding, probably *via* H-bond interactions and/or fixing the conformation of the 5-phosphate. Least important is the 2-hydroxyl; this can be changed to equatorial or even deleted with little loss of activity and introduction of large substituents at this position is tolerated.

Most modifications of the Ins(1,4,5)P_3 molecule have resulted in reduced potency in terms of Ca^{2+} release. However, in 1994, the discovery of the adenophostins (figure **3**) was reported [Takahashi *et al*, 1994]. These molecules, isolated from the culture broth of *Penicillium brevicompactum*, were reported to be around 100-fold more potent agonists at the Ins(1,4,5)P_3 receptor than Ins(1,4,5)P_3 itself in cerebellar microsomes. Although at first glance the molecule looks markedly different to Ins(1,4,5)P_3, it does bear the essential pharmacophore, the vicinal phosphates, and they are in a six-membered ring. Further, it has a hydroxyl at the position next to the phosphates, mimicking the 6-hydroxyl of Ins(1,4,5)P_3 and its third phosphate may enhance binding in the same way that the 1-phosphate of Ins(1,4,5)P_3 is thought to. The steric bulk of the adenosine moiety suggests that the receptor has a vacant area in this region. At what position this may be analogous to in Ins(1,4,5)P_3 itself depends upon the orientation in which adenophostin binds. The adenophostins are resistant to 5-phosphatase and 3-kinase and have prolonged activity as a result.

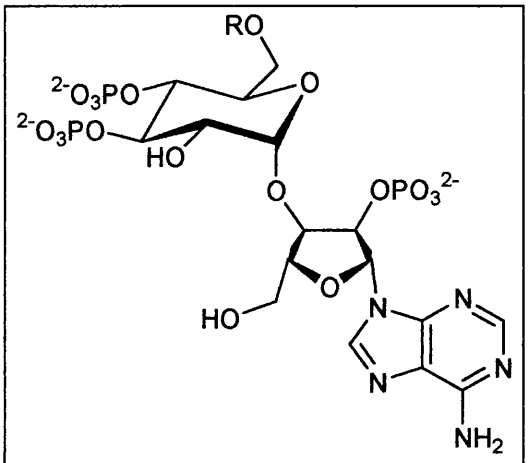


Figure 3 The adenophostins (R= H adenophostin A, R= Ac adenophostin B)

3.6 Ins(1,4,5)P_3 Receptor Antagonists

At present, only a small number of Ins(1,4,5)$_3$ receptor antagonists are known. Most potent are the polysulfated polysaccharide heparin and decavanadate ($V_{10}O_{28}^{6-}$) [Potter and Lampe, 1995], both however have low specificity. Most inositol phosphate analogues that have shown any antagonistic activity have also shown some Ca^{2+} releasing activity, the exception being the 5-methylphosphonate analogue of Ins(1,4,5)P_3.

The polyamines putrescine **1**, spermidine **2** and spermine **3** (Figure **4**), naturally present in all cells, have been shown to inhibit Ins(1,4,5)P_3-induced Ca^{2+} release in rat cerebellar microsomes, [Sayers and Michelangeli, 1993] possibly by an effect on the Ins(1,4,5)P_3 receptor.

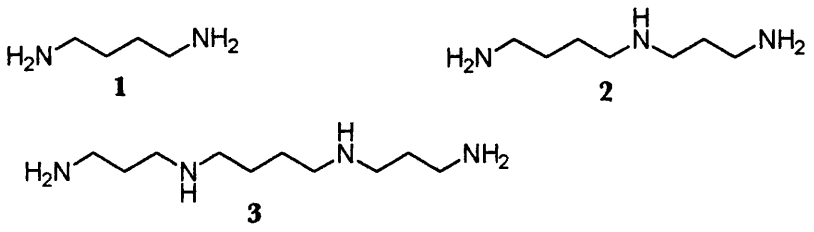
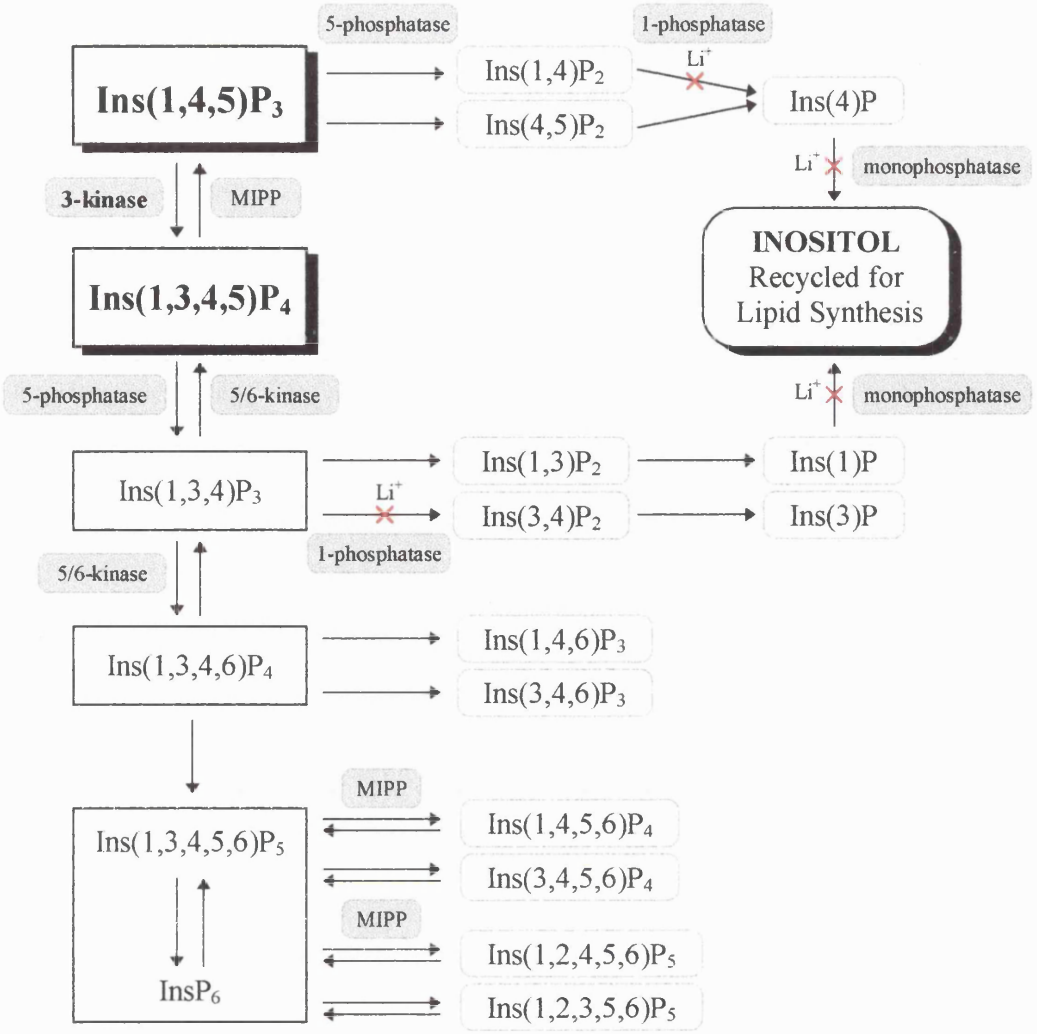


Figure 4 Putrescine, Spermidine, Spermine

Their potency in inhibiting Ca^{2+} release was related to the number of amino groups (and hence the number of charges), the order being spermine > spermidine > putrescine. This paralleled their ability to inhibit [^{3}H]Ins(1,4,5)P_3 binding. Under the conditions used for the Ca^{2+} release experiments, the inhibition of Ca^{2+} release was shown not to be due to the formation of a complex between Ins(1,4,5)P_3 and spermine as, at 1mM, spermine inhibited Ins(1,4,5)P_3-induced Ca^{2+} release by 50%, but spermine-agarose bound essentially no Ins(1,4,5)P_3 at that concentration. This suggested that the effect of spermine on Ins(1,4,5)P_3-induced Ca^{2+} release was due to an effect on the Ins(1,4,5)P_3 receptor. However, the study could not show that inhibition of [^{3}H]Ins(1,4,5)P_3 binding was not due to the formation of a complex between the negatively charged phosphates and the positively charged amines.

3.7 Metabolism of Ins(1,4,5)P_3

The action of Ins(1,4,5)P_3 must be terminated so that the cell is free to respond to further signals. Its metabolism (summarised in scheme **2**) is complex, generating many other inositol phosphates, the roles of which are the subject of much debate. The two most important enzymes involved in Ins(1,4,5)P_3 metabolism are inositol polyphosphate 5-phosphatase and D-*myo*-Inositol 1,4,5-trisphosphate 3-kinase. A recent study [Sims and Allbritton, 1998], suggests that the rate and route of metabolism of Ins(1,4,5)P_3 in *Xenopus laevis* oocytes is dependent upon Ins(1,4,5)P_3 concentration and intracellular free Ca^{2+} concentration. The rate and route of metabolism of [^{3}H]-Ins(1,4,5)P_3 was measured in whole oocytes (by microinjection) and in cytoplasmic extracts. At low Ins(1,4,5)P_3 concentration (100nM) and high Ca^{2+} concentration (≥1μM), Ins(1,4,5)P_3 was metabolised predominantly by 3-kinase, whereas at high Ins(1,4,5)P_3 concentration (8 μM or greater) 5-phosphatase was dominant irrespective of Ca^{2+} concentration. At Ins(1,4,5)P_3 and Ca^{2+} concentrations below 400nM the activities of 5-phosphatase and 3-kinase were comparable.



Scheme 2 Summary of inositol polyphosphate metabolism

Inositol Polyphosphate 5-Phosphatases

Inositol polyphosphate 5-phosphatases are a group of isoenzymes that dephosphorylate $Ins(1,4,5)P_3$ to inactive $Ins(1,4)P_2$ and can, therefore, be regarded as an effective way of deactivating the $Ins(1,4,5)P_3$ signal. There seem to be both cytosolic and membrane-bound forms of these enzymes [Verjans *et al*, 1994]. They appear to be relatively non-selective; many inositol phosphates are a substrate for these enzymes including inositol phospholipids, although different isoforms may show different degrees of substrate specificity. The first known example of an inborn defect in inositol polyphosphate metabolism involves the 5-phosphatases. The gene defective in Lowe's oculocerebrorenal syndrome, an X-chromosome linked condition characterised by protean manifestations including mental retardation, numerous eye abnormalities and impaired renal function, was found to encode a protein highly homologous to an isoform of 5-phosphatase [Attree *et al*, 1992]. This protein was subsequently produced

recombinantly [Zhang *et al*, 1995] and, was indeed, found to carry out the reactions of 5-phosphatase. Interestingly, it hydrolyses the phospholipid substrate (*i.e.* PtdIns(4,5)P_2 to PtdIns(4)P) 10- to 30-fold better than 5-phosphatase II, an isoform from human platelets; hence the protein is mainly a lipid 5-phosphatase.

There has been success in the synthesis of potent, selective, non-Ca^{2+} mobilising inhibitors of 5-phosphatase, some examples of which are shown in figure **5**. The most potent 5-phosphatase inhibitor to date is L-*chiro*-2,3,5-trisphosphorothioate **4** (K_i = 230nM†) [Safrany *et al*, 1994] however it is also a partial agonist at the Ins(1,4,5)P_3 receptor and a potent inhibitor of 3-kinase. L-*myo*-Inositol 1,4,5-trisphosphorothioate inhibits 5-phosphatase **5** (K_i = 430nM) showing 250-fold selectivity over 3-kinase and the *meso*-compound *myo*-inositol 1,3,5-trisphosphorothioate **6** (K_i = 520nM) shows 475-fold selectivity. More potent, however, is L-*chiro*-inositol 1,4,6-trisphosphorothioate **7** (K_i = 300nM) and this compound is also the most selective; it interacts neither with the receptor nor with 3-kinase.

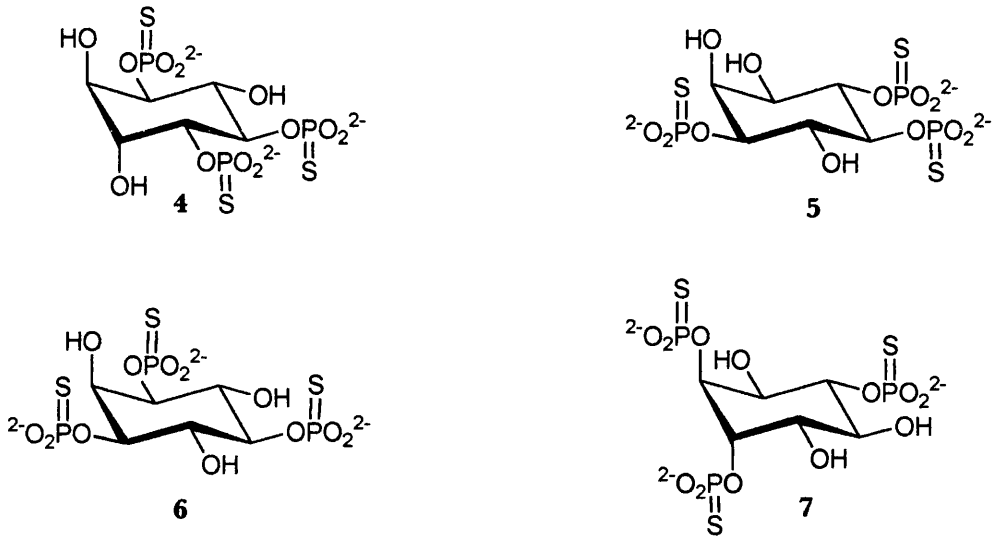


Figure 5 Inhibitors of Inositol Polyphosphate 5-Phosphatase

Ins(1,4,5)P_3 3-kinase

D-*myo*-Inositol 1,4,5-trisphosphate 3-kinase catalyses the phosphorylation of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4. The roles of 3-kinase and its product, Ins(1,3,4,5)P_4, are pertinent to the work described in chapter 3 and will be discussed in detail in that chapter.

Multiple Inositol Polyphosphate Phosphatase (MIPP)

Ins(1,3,4,5)P_4 and higher inositol phosphates are metabolised by MIPP. The enzyme can hydrolyse the 6-phosphate of Ins(1,3,4,5,6)P_5 and Ins(1,4,5,6)P_4, and the 3-phosphate of

† Human erythrocyte ghost 5-phosphatase-catalysed breakdown of [^{3}H]-Ins(1,4,5)P_3 [Safrany *et al*, 1994].

Ins(1,3,4,5)P_4. Ins(1,3,4,5,6)P_5 can be formed independently of phospholipase C by direct phosphorylation of inositol [Stephens and Irvine, 1990] and MIPP may function to produce Ins(1,4,5)P_3, independently of phospholipase C activity. This has been demonstrated in *Dictyostelium* cell homogenates and by a single enzyme purified from rat liver [Van Dijken *et al*, 1995]. The presence of MIPP in cell preparations used to study the actions of Ins(1,3,4,5)P_4 may lead to misleading results, since some observed effects may be due to Ins(1,4,5)P_3 produced by the action of MIPP on applied Ins(1,3,4,5)P_4. This problem has been overcome by using the 3-phosphorothioate analogue of Ins(1,3,4,5)P_4 that is resistant to MIPP [Wilcox *et al*, 1993b], by using a MIPP inhibitor such as $InsP_6$ [Wilcox *et al*, 1993b] or by conducting experiments at 4°C which arrests this metabolism [Gawler *et al*, 1990].

Inositol 1,3,4-trisphosphate 5/6-kinase

Ins(1,3,4)P_3 is phosphorylated at the 5- or 6-position by inositol 1,3,4-trisphosphate 5/6-kinase. The enzyme, which has been purified and characterised from rat liver, has a high affinity for Ins(1,3,4)P_3 and is about 5 times more active towards the 6-phosphate than towards the 5-phosphate [Abdullah *et al*, 1992]. The enzyme is not affected by Ca^{2+}/calmodulin or protein kinase A or C. It is not known whether its products have signalling functions or whether they are just precursors to inositol pentakisphosphates and hexakisphosphate.

4 Aims and objectives of this work

The aim of the work within the first part of this thesis was the synthesis of Ins(1,4,5)P_3 *via* a rapid, novel route and the synthesis of an Ins(1,3,4,5)P_4 analogue for use in biological studies. It was intended to explore the partial hydrolysis of the orthoacetate orthoester of *myo*-inositol to yield acetate ester-protected intermediates. The reaction would be carried out on camphanate derivatives that allow a chiral desymmetrisation of the molecule, such that the natural enantiomer of Ins(1,4,5)P_3 would be obtained. The goal was to see if the reaction could be controlled in such a way that a useful yield of a direct precursor to Ins(1,4,5)P_3 could be obtained. Emphasis was placed upon ease and rapidity of synthesis and consideration given to the possibility of obtaining other useful products of the orthoester partial hydrolysis. The second overall target was the Ins(1,3,4,5)P_4 analogue having a hydroxymethyl phosphate at the 3-position. This was to be used as a standard in biological studies involving Ins(1,4,5)P_3 3-kinase. The molecule may have interesting properties as an Ins(1,3,4,5)P_4 surrogate and may prove useful in the synthesis of a potential multisubstrate analogue inhibitor of Ins(1,4,5)P_3 3-kinase.

Chapter 2:
Synthesis of D-*myo*-Inositol 1,4,5-Trisphosphate

1 Introduction

Chapter 1 gave an overview of the significance of Ins(1,4,5)P_3 in animal cell physiology. It can be seen that we still have much to learn about the details of this ubiquitous pathway. Thus there is continuing demand for supplies of pure homochiral Ins(1,4,5)P_3. Many synthetic routes to Ins(1,4,5)P_3 have been published; they are generally time-consuming, long, linear sequences involving extensive chromatography. Hence, a practical, expeditious synthesis would be welcome.

2 Synthesis of Inositol Phosphates: General Considerations

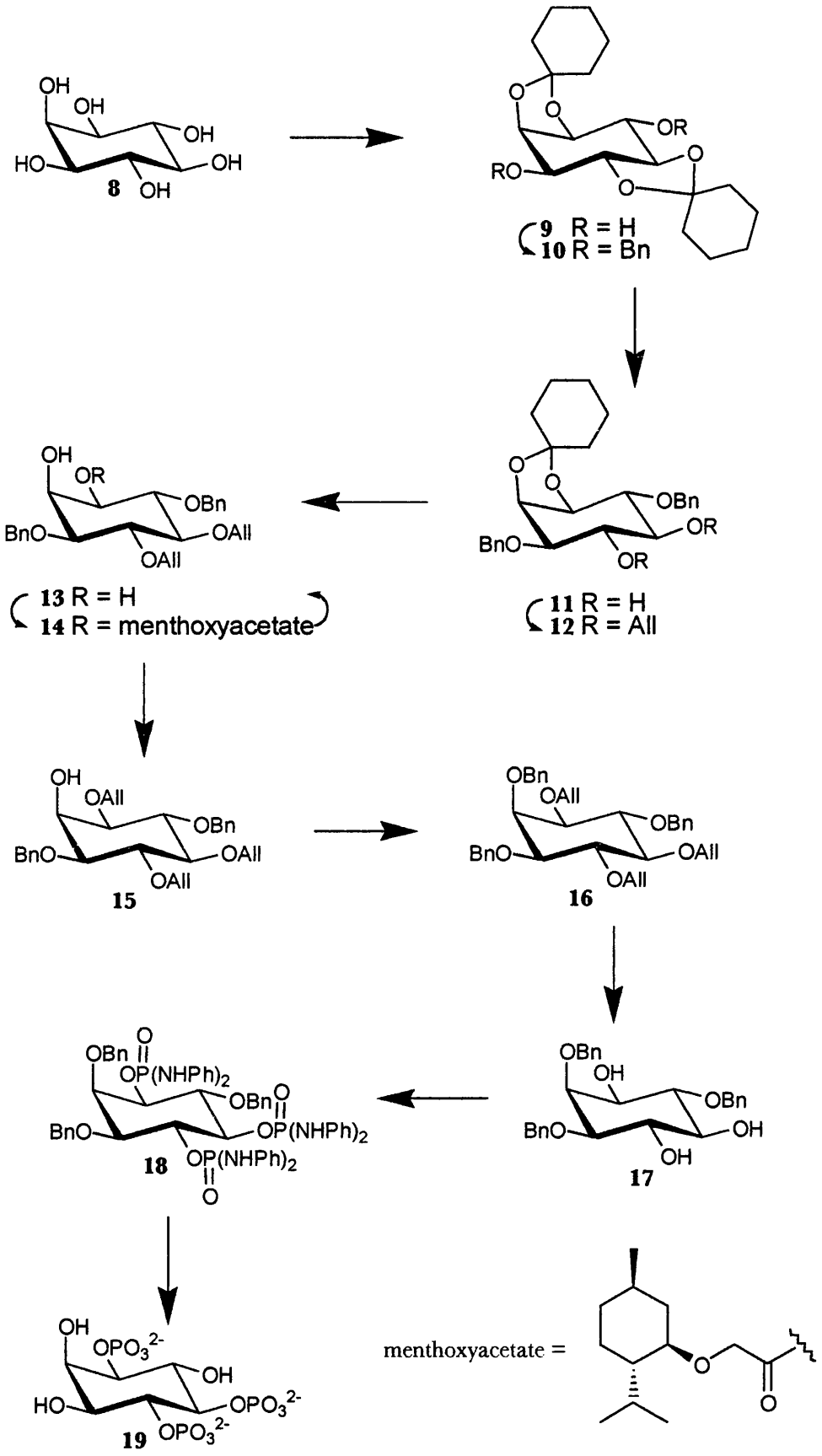
When one is designing a synthesis of an inositol phosphate from *myo*-inositol consideration must be paid to a number of key points, namely:

- The selective protection of hydroxyl groups. Much attention has been paid to this and there are now a large number of protection strategies.
- Different enantiomers may show vastly different biological action, so the preparation of homochiral material is highly desirable. Resolution usually involves preparation of a pair of diastereoisomers at a convenient point in the synthesis and separation of these by chromatography or recrystallisation. This is usually relatively straightforward, although it may involve additional protection and deprotection steps.
- Once a suitably protected derivative has been prepared, the free hydroxyls are phosphorylated. Phosphorylation can be performed using phosphorus-V reagents or phosphorus-III reagents. Phosphorus-V reagents are more stable but less reactive and more likely to form cyclic phosphates when phosphorylating vicinal diols. Phosphorus-III chemistry involves phosphitylation followed by oxidation.
- Protected hydroxyls and phosphates must be deprotected in a manner that avoids phosphate migration. This may require a one or two step process.
- Purification of the final compound is of great importance since many inositol phosphates are active at very low concentrations. A tiny contamination with a more active isomer may give misleading biological results. Final purification by HPLC or ion-exchange chromatography is highly desirable.

These points will be illustrated by the chemistries described within this chapter.

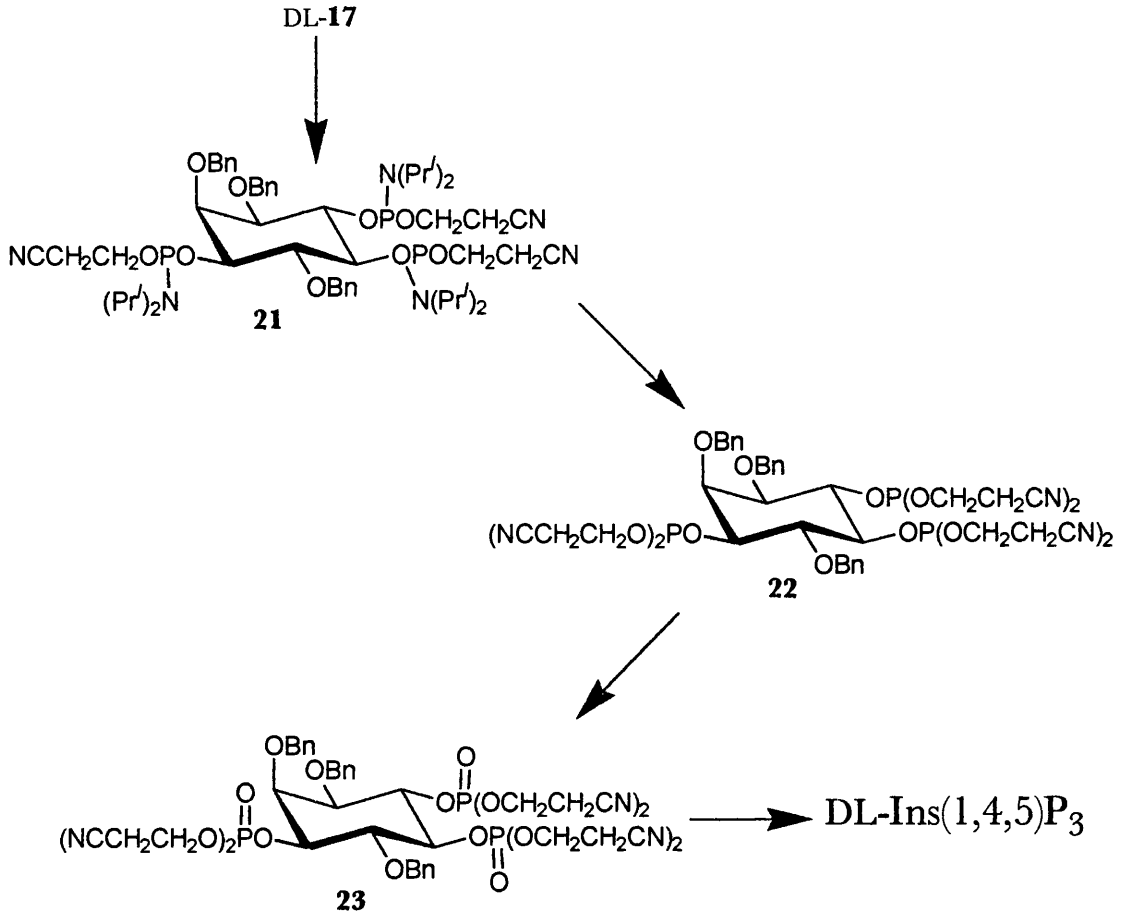
3 Previous Syntheses of Ins(1,4,5)P_3

The first total synthesis of Ins(1,4,5)P_3 was by Ozaki and co-workers in 1986 [Ozaki *et al*, 1986]. The synthesis is illustrated in scheme **3**. DL-1,2:4,5-Di-*O*-cyclohexylidene-*myo*-inositol **9**, prepared from *myo*-inositol **8**, was benzylated and the *trans*-4,5-acetal was cleaved selectively to yield diol **11**. Allylation, then hydrolytic removal of the remaining acetal, gave diol **13**. The authors chose to resolve the molecule at this stage by making the L-menthoxyacetate ester at the 1-position. The diastereoisomers were separated by recrystallisation, followed by chromatography of the mother liquor, to afford D-**14** in 39% yield. The menthoxyacetate ester was cleaved and the diol was allylated regioselectively. Benzylation followed by deallylation gave the key intermediate D-2,3,6-tri-*O*-benzyl-*myo*-inositol **17**. Although long (10 steps), the synthesis to this point is relatively efficient and straightforward; subsequent phosphorylation and deprotection, however, are low yielding. The triol **17** was phosphorylated with dianilidophosphoric chloride to yield **18** in *ca.* 41% yield. Treatment of **18** with isoamyl nitrite in pyridine/AcOH/Ac_2O removed the protecting groups from phosphorus, then hydrogenolysis over Pd removed the benzyl groups to give Ins(1,4,5)P_3 **19**. No indication as to the purity of the final product is given in the paper.



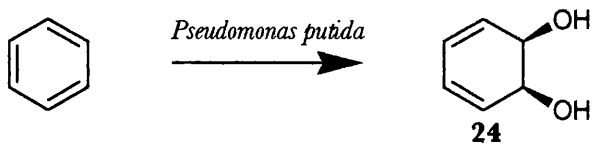
Scheme 3 The synthesis of Ins(1,4,5)P_3 by Ozaki *et al* [Ozaki *et al*, 1986].

A synthesis of racemic Ins(1,4,5)P_3 from racemic **17**, using an improved phosphorylation strategy [Cooke and Potter, 1987], was soon developed; this is illustrated in scheme **4**. The triol **17** was treated with diisopropylamino(2-cyanoethyl)chlorophosphine to give the phosphite triester **21** in 90% yield. This was converted to the di-(2-cyanoethyl)phosphite derivative **22**, then quantitatively oxidised to the protected phosphate **23** using *t*-butyl hydroperoxide. All the protecting groups were removed in one step, employing sodium in liquid ammonia, to afford racemic Ins(1,4,5)P_3 after ion exchange chromatography.



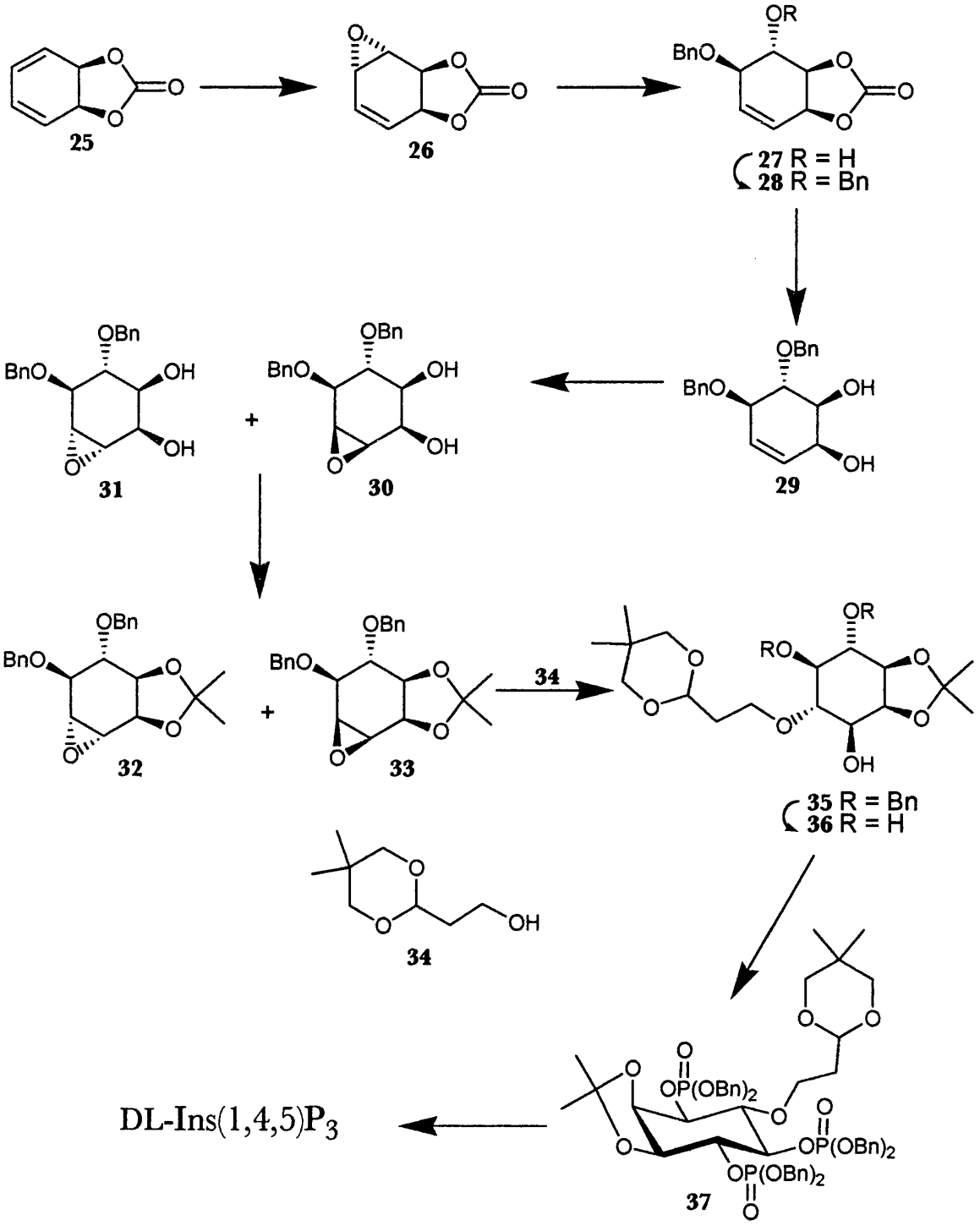
Scheme 4 Synthesis of racemic Ins(1,4,5)P_3 from racemic **17**.

These syntheses illustrate the typical approaches to inositol polyphosphate synthesis from *myo*-inositol. Other starting materials can be employed. For example, the need for optical resolution can be negated by the use of chiral starting materials, such as L-quebrachitol or (–)-quinic acid. Interestingly, a synthesis of Ins(1,4,5)P_3 was devised starting from benzene [Ley *et al* 1990]. The synthesis starts with *cis*-1,2-dihydroxycyclohexa-3,5-diene **24**, a product of the microbial oxidation of benzene (scheme **5**).



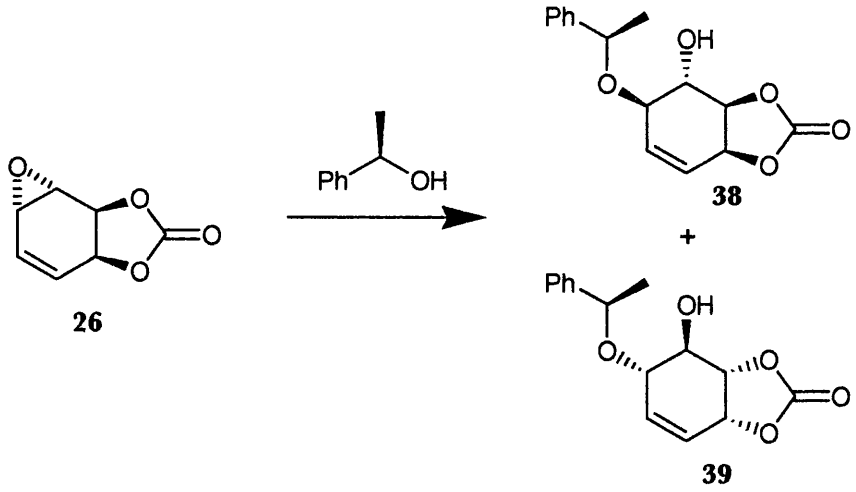
Scheme 5 Oxidation of benzene by *Pseudomonas putida.*

The remaining hydroxyls were introduced by stereoselective epoxidation of the alkenic bonds then regioselective ring opening. The synthesis is illustrated in scheme **6**.



Scheme 6 The synthesis of racemic Ins(1,4,5)P_3 *via* stereoselective oxidation of alkenic bonds.

Thus the cyclic carbonate **25** was prepared from **24** with sodium methoxide and dimethyl carbonate. Epoxidation with 3-chloroperoxybenzoic acid (MCPBA) gave the α-epoxide **26** in 4.6:1 excess over its β-isomer. Regioselective ring opening of the epoxide of **26** with benzyl alcohol under camphorsulfonic acid catalysis gave **27** which was benzylated. Basic hydrolysis of the cyclic carbonate gave diol **29** where the hydroxyl groups are placed for directed epoxidation. Thus epoxidation with MCPBA proceeded to give **30** and **31** in a 9:1 ratio. The authors found it more convenient to make acetonides **32** and **33** before separating the α- and β-epoxides by flash chromatography. The epoxide was opened with alcohol **34** (which was synthesised in two steps) as its sodium salt in hexamethylphosphoramide/THF‡. This gave the required regioisomer **35** in 43% yield. Hydrogenolysis removed the benzyl groups to give **36** which was phosphorylated with *n*-butyllithium/tetrabenzylpyrophosphate to give **37**. Hydrogenolysis, then acidic hydrolysis removed the protecting groups to give racemic Ins(1,4,5)P_3. This is a long synthetic sequence involving extensive chromatography (7 of the 11 steps from diol **24** involve chromatography, in addition, both steps for production of **34** required chromatography) that provides racemic Ins(1,4,5)P_3 only. However, the route was readily modified to provide homochiral material by a method that did not add any more steps to the sequence (scheme **7**).

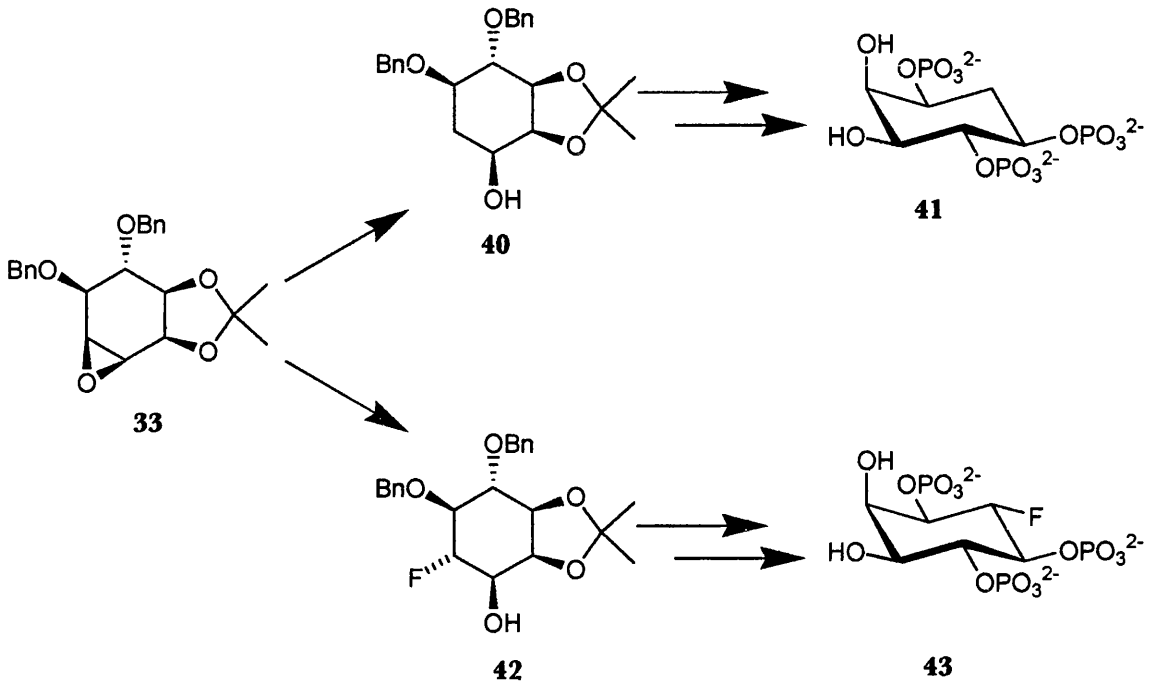


Scheme 7 Epoxide ring opening using a chiral nucleophile gives diastereoisomers **38** and **39**, intermediates for the synthesis of both enantiomers of Ins(1,4,5)P_3.

Epoxide **26** was treated with (R)-(+)-1-phenylethanol with $HBF_4.OEt_2$ catalysis to give diastereoisomeric alcohols **38** and **39** which were separated by HPLC. These led to D- and L-Ins(1,4,5)P_3 *via* similar methods employed to those for the racemic route. The absolute

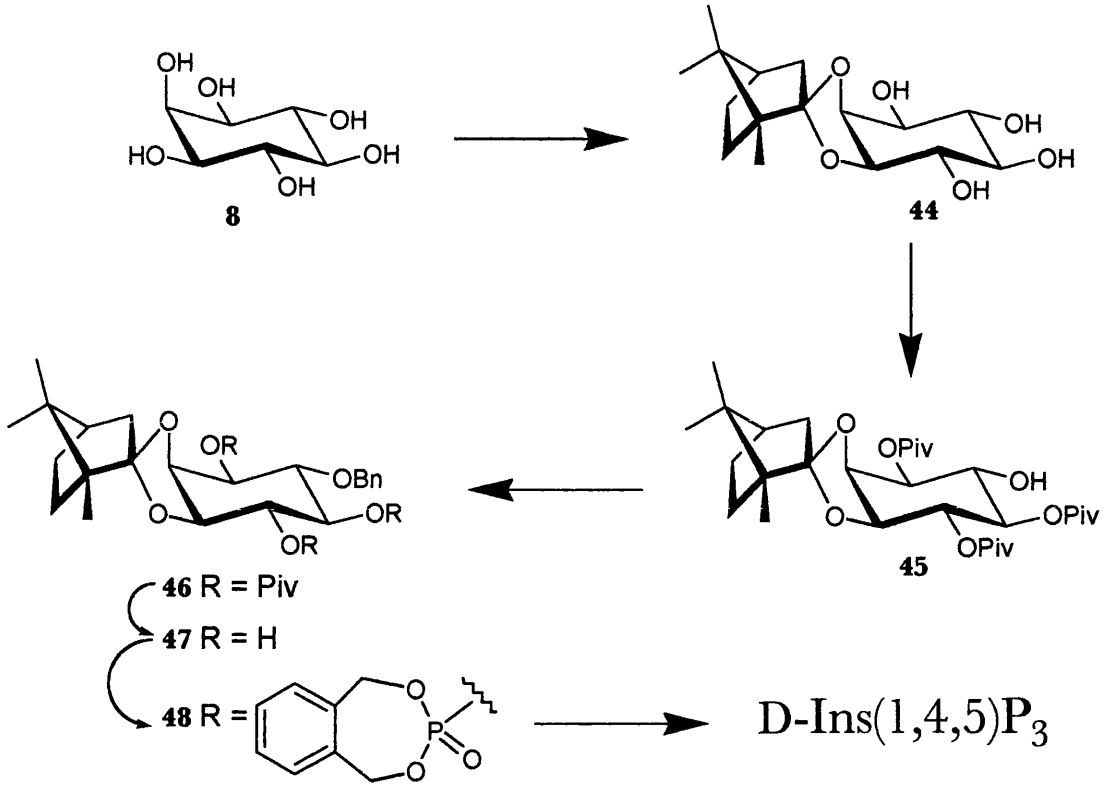
‡ The use of this reagent as a hydroxide equivalent allowed deprotection by acidic hydrolysis of the acetal; this generates a β-alkoxy aldehyde which spontaneously undergoes β-elimination to yield the free hydroxyl group.

configuration of **38** and **39** was determined by X-ray crystallography of a later derivative. In addition to the synthesis of Ins(1,4,5)P_3, intermediates in this synthesis were useful in the synthesis of other inositol polyphosphates (scheme **8**). Notably, epoxide **33** was opened with $LiAlH_4$ to furnish **40**, which was transformed into 6-deoxy-Ins(1,4,5)P_3 **41**; ring opening with tris(dimethylamino)sulfur (trimethylsilyl)difluoride gave **42**, which was transformed into another 6-position modified analogue 6-deoxy-6-fluoro-Ins(1,4,5)P_3 **43**.



Scheme 8 Compound **33**, a versatile intermediate for the synthesis of 6-position modified Ins(1,4,5)P_3 analogues.

There are at least 15-20 more reported syntheses of Ins(1,4,5)P_3. Probably the shortest route is that of Salamonczyk and Pietrusiewicz [Salamonczyk and Pietrusiewicz, 1991; Pietrusiewicz *et al*, 1992] which is illustrated in scheme **9**.



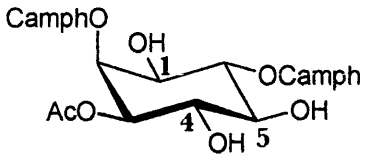
Scheme 9 The synthesis of Ins(1,4,5)P_3 by Salamonczyk and Pietrusiewicz [Salamonczyk and Pietrusiewicz, 1991]

The first step was a difficult precipitation-driven equilibration reaction, where the complex mixture of mono- and bis- acetals, formed from *myo*-inositol **8** and D-camphor dimethyl acetal under sulfuric acid catalysis in DMSO, was subjected to hydrolytic conditions ($CHCl_3$/MeOH/H_2O 50:5:1 and PTSA) leading to the formation of the single product **44** which precipitated gradually from the reaction mixture in 65% yield. The conditions for this reaction undoubtedly require very careful control. The authors claim that only tiny amounts of free inositol or other monoacetals are detectable in the product. The absolute configuration was determined by converting **44** to its tetrabenzyl derivative, a known compound, as well as by X-ray analysis of intermediate **46**. Treatment of **44** with 4 equivalents of pivaloyl chloride gave the tripivaloyl derivative **45** in 48% yield after chromatography. Benzylation, then basic hydrolysis of the pivaloate esters, gave triol **47** in 46% yield from **45**. Phosphitylation then oxidation gave the protected phosphate **48**. Deprotection was carried out in two stages. Hydrogenolysis of the benzyl groups, then acid hydrolysis of the acetal with acetic acid, gave Ins(1,4,5)P_3, which was isolated by precipitation in methanol as its sodium salt. It was not indicated whether or not further purification was carried out or whether the Ins(1,4,5)P_3 was evaluated biologically.

4 A Rapid and Practical Synthesis of D-*myo*-Inositol 1,4,5-Trisphosphate

4.1 Overview

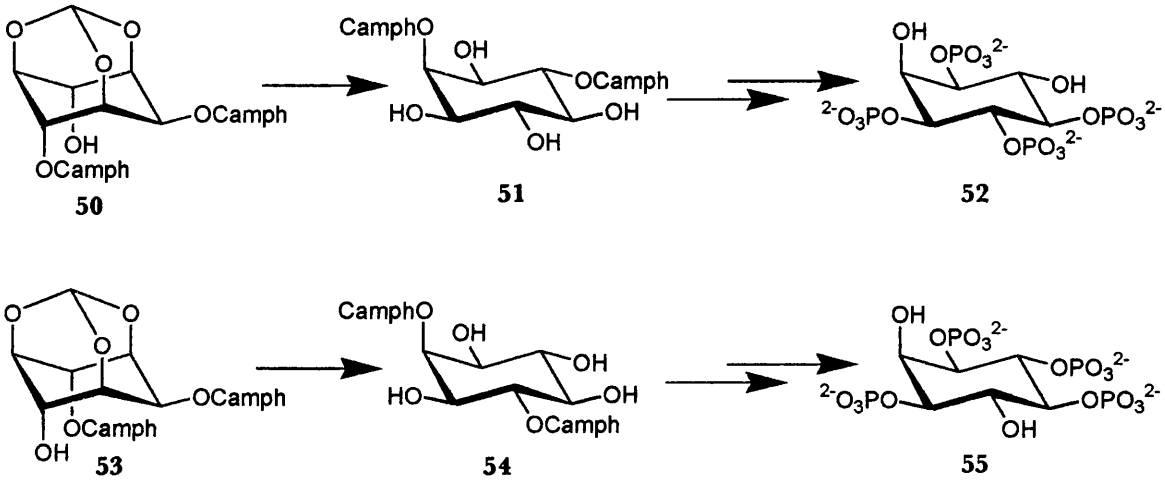
The triol **49** was proposed as the key intermediate for the rapid synthesis of Ins(1,4,5)P_3.



49

This intermediate was chosen for two reasons:

(*i*) A colleague had previously synthesised diastereoisomeric di-camphanates of *myo*-inositol orthoformate **50** and **53**, separable by chromatography, and utilised these for a rapid synthesis of both enantiomers of Ins(1,3,4,5)P_4 **52** and **55**, scheme **10** [Riley *et al*, 1997].

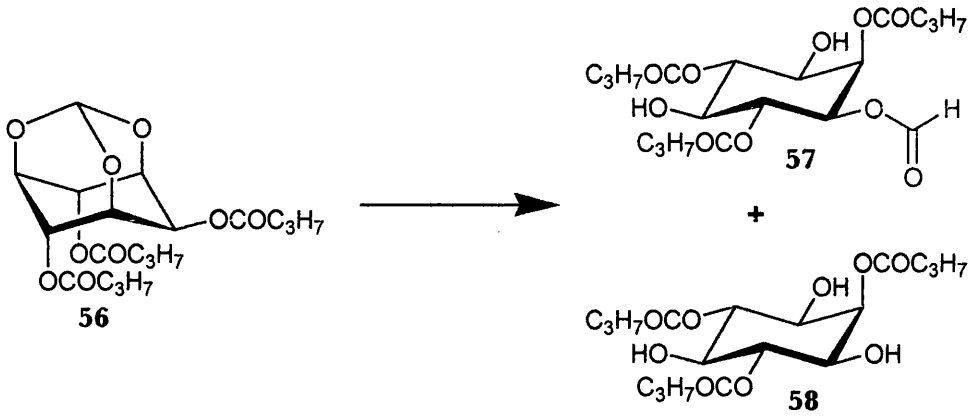


Scheme 10 Synthesis of both enantiomers of Ins(1,3,4,5)P_4, Riley *et al* [Riley *et al*, 1997].

It can be seen that additional protection at the 3-position in **51** would yield an intermediate such as **49** that would yield Ins(1,4,5)P_3 upon phosphorylation and deprotection.

(*ii*) During routine hydrolysis (using HCl/MeOH at reflux for example) of intermediates with orthoformate protection, small amounts of formate esters as products of partial deprotection had often been observed on TLC. These intermediates usually disappeared as the reaction progresses and, if not, are easily lost upon treatment with concentrated aqueous ammonia. Orthoformate hydrolysis under milder conditions allowed the formation of enough formate ester to allow convenient isolation by flash chromatography [Lampe, 1993]. 2,4,6-Tri-*O*-

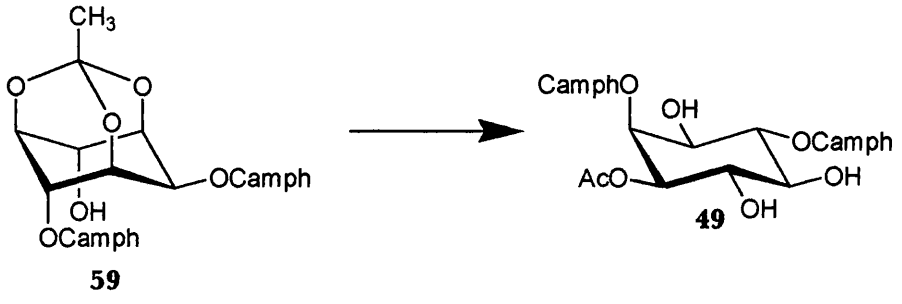
butanoyl-*myo*-inositol orthoformate **56** was treated with 80% aqueous trifluoroacetic acid (TFA) and 2,4,6-tri-*O*-butanoyl-1-*O*-formyl-*myo*-inositol **57** was formed as a minor product (14%), this is shown in scheme **11**.



Scheme 11 Orthoformate hydrolysis to yield a formate ester

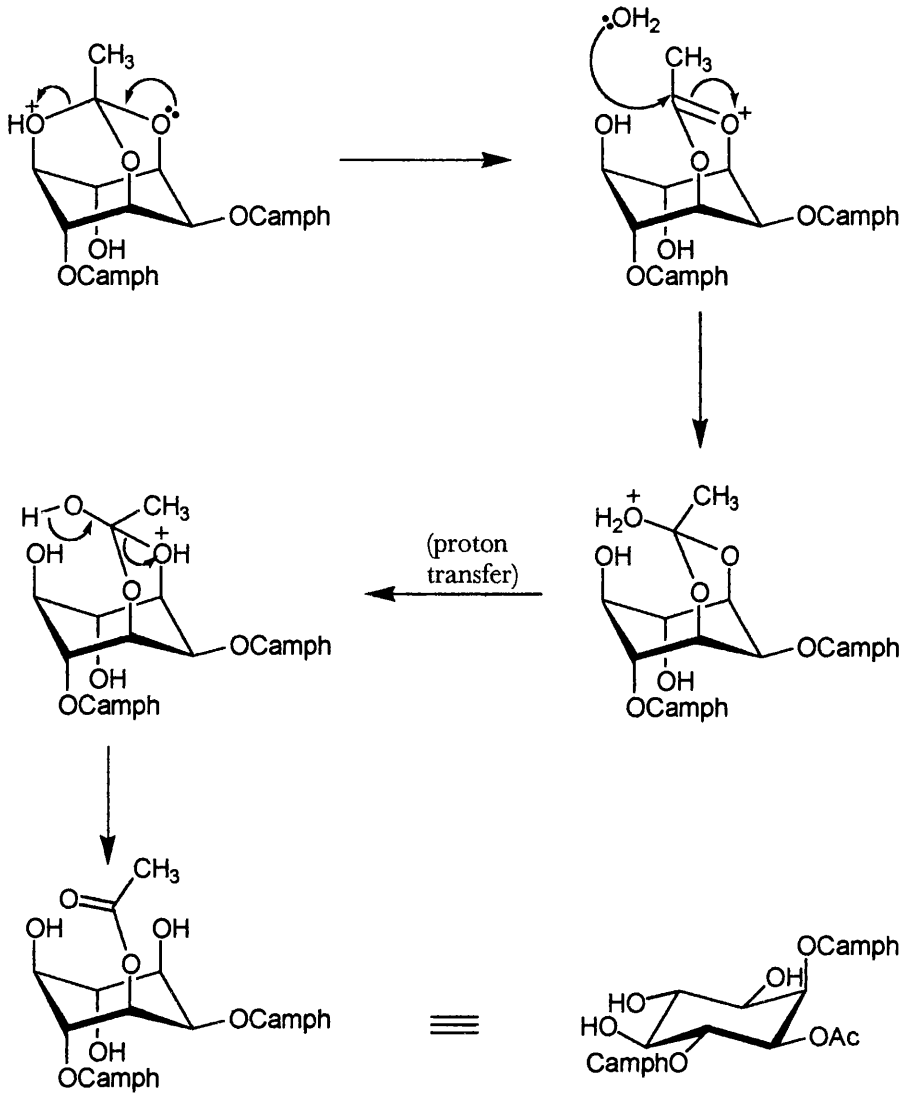
Although these formate esters were not sufficiently stable to be useful synthetically, this finding did suggest that a different, more stable orthoester, such as an orthoacetate, might be useful as an intermediate protecting group that could be later transformed by partial hydrolysis into an acetate ester of defined regiochemistry.

Thus, considering these two points, the strategy for the synthesis of **49** was first to synthesise **59** (scheme **12**) from *myo*-inositol orthoacetate, then to establish reaction conditions that allow only partial hydrolysis of the orthoacetate functionality, such that acetate protection is retained at the 3-position.



Scheme 12 Partial hydrolysis of *myo*-inositol orthoacetate derivative **59**

This reaction would probably proceed *via* the mechanism shown in scheme **13**.



Scheme 13 Mechanism of partial hydrolysis of a *myo*-inositol orthoacetate derivative. Only one of the potentially similar pathways is shown for simplicity.

Examining this mechanism, it is reasonable to assume that the reaction should yield a mixture of 1-, 3-, and 5-acetate esters **60, 49** and **61** (Figure **6**).

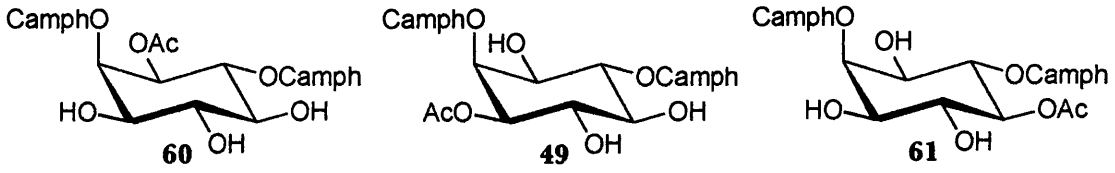
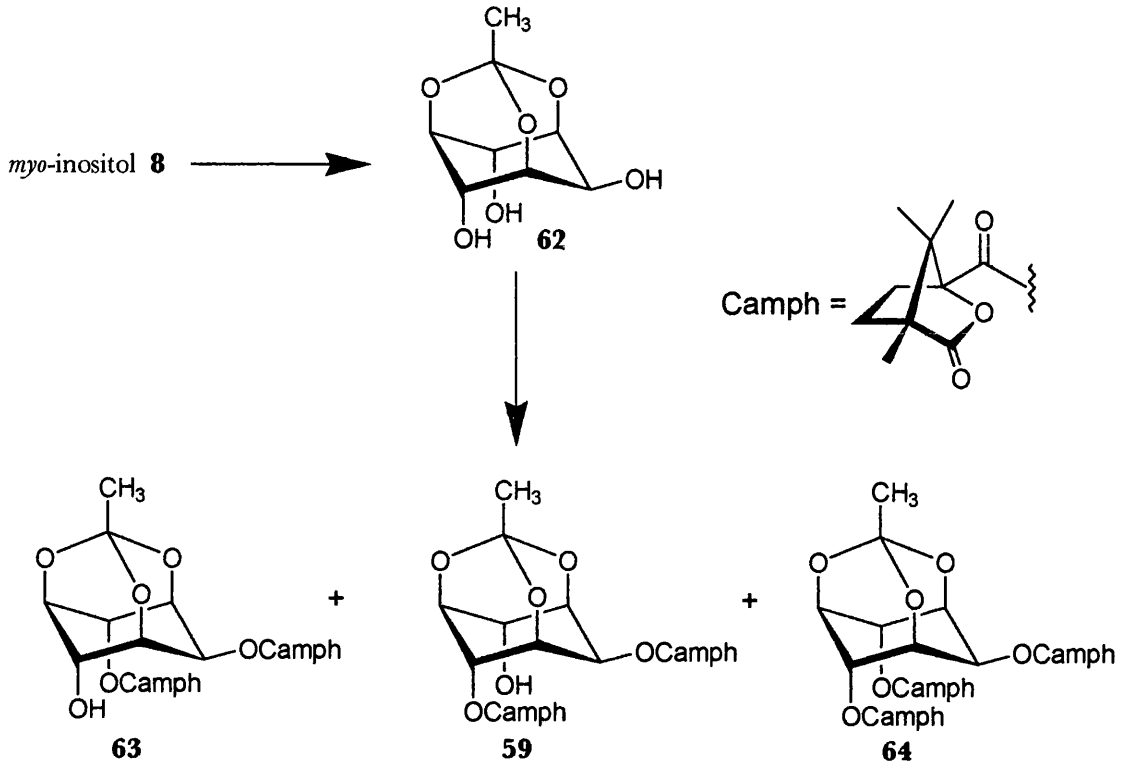


Figure **6** Possible regioisomers from partial hydrolysis of **59**.

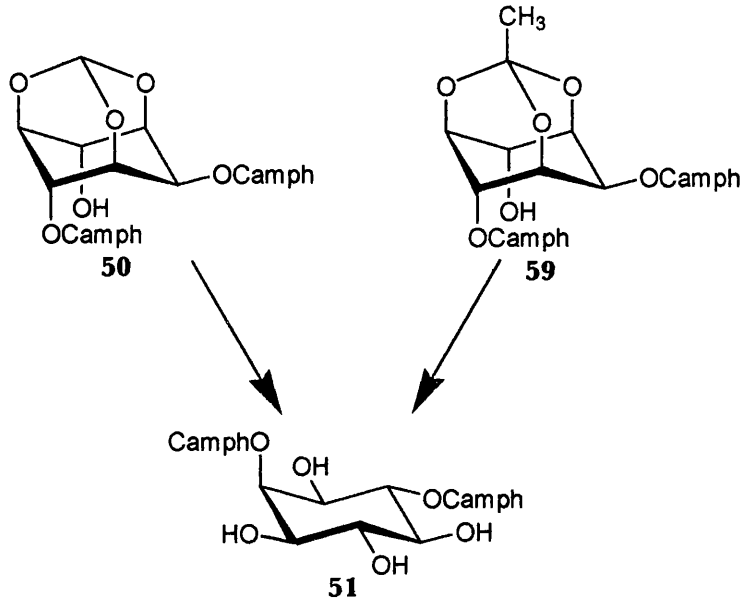
4.2 Synthesis of the Diastereoisomeric Dicamphanates of *myo*-Inositol Orthoacetate



Scheme 14 Synthesis of camphanate esters of *myo*-inositol orthoacetate from *myo*-inositol

The first unambiguous synthesis of an orthoester of *myo*-inositol was the synthesis of the orthoformate orthoester, reported in 1985 [Lee and Kishi, 1985]. Subsequently, this intermediate has found wide application in inositol phosphate synthesis [for example: Baudin *et al*, 1988; Billington *et al*, 1989; Ozaki *et al* 1994]. Orthoformate protection is relatively easy to introduce by treatment of *myo*-inositol with triethyl orthoformate in DMF with PTSA as a catalyst, it is stable under many reaction conditions and it is easily removed by acidic hydrolysis. Thus it would be expected that using triethyl orthoacetate in place of triethyl orthoformate in an analogous reaction would give rise to orthoacetate protected *myo*-inositol. Indeed, this is the case and *myo*-inositol orthoacetate **62** (scheme **14**) was easily prepared from *myo*-inositol. Triethyl orthoacetate was added to a suspension of *myo*-inositol and PTSA in DMF and heated at 140°C for three hours. Flash chromatography eluting with acetonitrile followed by recrystallisation from methanol gave **62** as colourless crystals in 57% yield. As already noted, the synthesis of diastereoisomeric di-camphanates of *myo*-inositol orthoformate has been reported [Riley *et al*, 1997]. It was found, not surprisingly, that the orthoacetate also underwent such chemistry. Treatment of **62** with 2 equivalents of (1*S*)-(−)-camphanoyl chloride and a catalytic amount of DMAP in dichloromethane gave the two diastereoisomeric di-camphanates

59 and **63** as well as some unwanted tri-camphanate **64**. These products were separated by flash chromatography and further purified by recrystallisation. At this stage, it was not possible to determine the regiochemistry of these diastereoisomers, *i.e.* which was the 2,6-diester and which was the 2,4-diester. Therefore, it was necessary to convert one of them to a known reference compound and to measure its optical rotation (or gain an X-ray crystal structure). A convenient reference compound was available [Riley *et al*, 1997]. In the synthesis of the enantiomers of Ins(1,3,4,5)P_4, Riley *et al* had determined the X-ray structure of **50** (scheme **15**) and subsequently converted it to **51**. By hydrolysing the orthoacetate functionality of one of the diastereoisomers **59** or **63** it would be possible to compare the optical rotation of the resulting tetrol with that of authentic material **51** and thus determine the absolute configuration.



Scheme 15 Tetrol **51** is a convenient reference compound to determine the absolute configuration of **59**.

This proved to be more difficult than anticipated and was achieved in only 9% yield by treating **59** in MeOH/aqueous HCl heated to reflux for 14 hours. The formation of considerably more polar products as shown by TLC, before complete orthoacetate removal, suggested that the low yield was due to extensive loss of camphanates. This illustrated the more robust nature of the orthoacetate compared to orthoformate. Orthoformate protection would typically be lost in less than one hour in MeOH/aqueous HCl under reflux. Hence, it would appear that the conditions required to cleave the orthoacetate completely are conditions that also cleave camphanate esters. However, despite the low yield, enough **51** was recovered to measure the optical rotation of the product and confirm the absolute configuration of the original compound **59**.

The reaction in scheme **14** yielded an excess of the 2,6-diester **59** over the 2,4-diester **63** when carried out in CH_2Cl_2. Carrying out the reaction in pyridine, however, gave an excess of the 2,4-diester (table **2**); this is in agreement with the findings of Riley [Riley, 1996] who carried out analogous reactions on the diesters of the orthoformate.

	Dichloromethane	**Pyridine**
2,4-diester **63**	24%	37%
2,6-diester **59**	34%	20%

Table 2 Yields of the diastereoisomeric dicamphanates in CH_2Cl_2 and in pyridine.

4.3 Synthesis of the key intermediate: 1D-3-Acetyl-2,6-Di-*O*-(–)-Camphanoyl *myo*-Inositol

The next stage was to attempt the synthesis of the key Ins(1,4,5)P_3 precursor **49**, from **59**. Repeating the conditions employed by Lampe [Lampe, 1993] in the synthesis of a formate ester by-product of orthoformate hydrolysis (scheme **11**), *i.e.* 80% aqueous trifluoroacetic acid, 4 hours at room temperature, had virtually no effect on the molecule. Elevation of the reaction temperature to reflux resulted in complete hydrolysis and loss of camphanates and, as described earlier, using aqueous HCl in MeOH gave similar results. Returning to 80% aqueous TFA at room temperature and lengthening the contact time proved to be more promising. It was found that treatment of **59** with 80% aqueous TFA at room temperature for 7 days gave 3 products as shown by TLC. One was the product of complete orthoacetate deprotection **51**, the other two having an Rf between that of **51** and that of the starting material, suggesting partial deprotection products. These were separated with some difficulty by flash chromatography eluting with EtOAc, then recrystallised. The ^1H NMR spectrum of the first product (figure **7**) showed 3 inositol ring proton signals shifted downfield suggesting that 3 hydroxyl groups were esterified. The inositol-H signal of one of the esterified positions (at δ 4.9) is a doublet of doublets (after D_2O exchange) showing a small axial-equatorial coupling and a larger axial-axial coupling. This must be position 1 or 3, since the only equatorial proton on the *myo*-inositol ring is at position 2; therefore the 1 or 3 acetate has formed. Further evidence was a singlet at δ 2.02, integrating for three protons; this is consistent with an acetate methyl signal. Of the other two signals corresponding to esterified positions, the signal at δ 5.3 is a broad triplet (after D_2O exchange), suggesting that this must correspond to position 6, as the signal for the proton at position 2 would show smaller equatorial-axial coupling only. Knowing the signal corresponding to the proton at position 6 it was possible to assign all the other signals from the ^1H-^1H coupled COSY spectrum (figure **8**) and thus define the regiochemistry of this first

product. The structure was confirmed as the 3-acetate ester, **49**. Note that the ^1H NMR spectrum of **49**, shown in figure **7**, illustrates beautifully, after D_2O exchange, the typical multiplicities seen for *myo*-inositol ring protons.

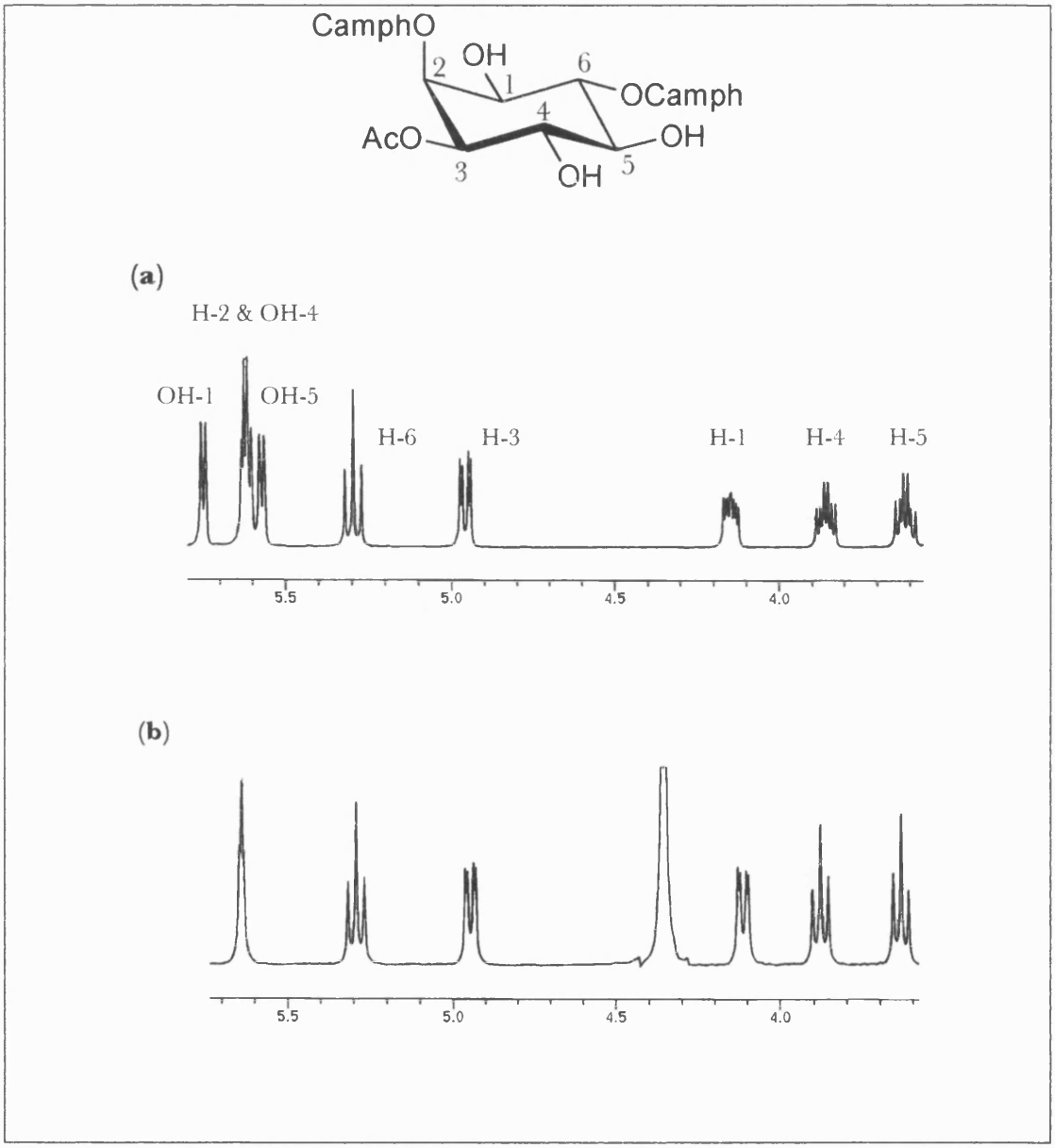


Figure 7 Section of the ^1H NMR spectrum of **49** showing the inositol ring protons (**a**) normal spectrum (**b**) D_2O exchange (400MHz, $[^2H]_7$-DMF)

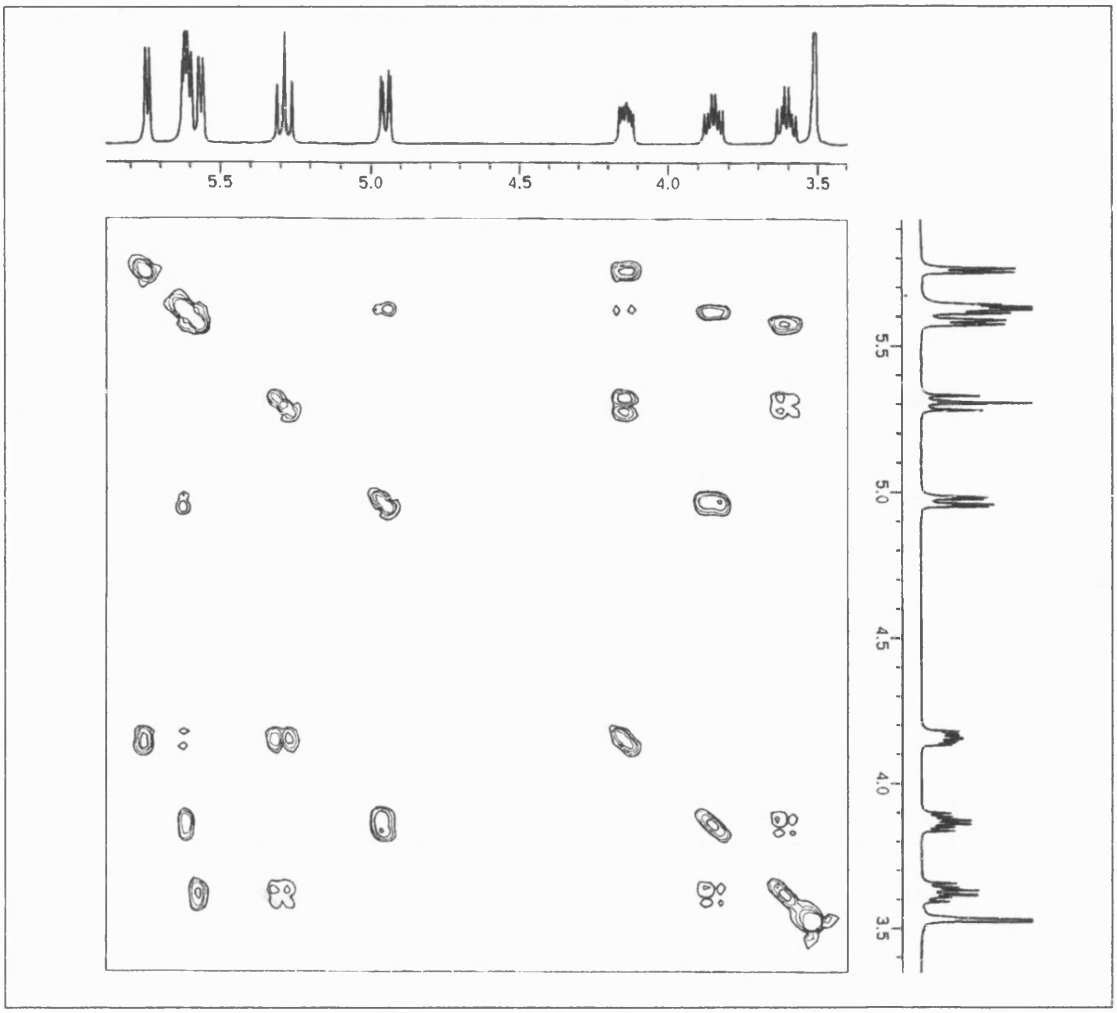


Figure 8 ^{1}H-^{1}H COSY spectrum of **49** (400MHz, $[^{2}H]_{7}$-DMF).

The next product was therefore expected to be either the 1-acetate **60** or the 5-acetate **61**. Analysis of its ^{1}H NMR spectrum and ^{1}H-^{1}H coupled COSY spectrum in a similar manner to that described for the 3-acetate showed it to be the 1-acetate ester **60**. No 5-acetate **61** formed in this reaction. This is in agreement with the findings of Lampe [Lampe, 1993] who saw no 5-formate in the partial hydrolysis of 2,4,6-tri-*O*-butanoyl-*myo*-inositol orthoformate. The reason for this cannot be predicted with certainty. It could be argued that in **59** the 5-oxygen is always the first to be protonated upon treatment with acid and therefore will always lose its orthoester bond. However there appears to be no obvious reason as to why this should be the case as there is probably no difference in the pKa values of these oxygens. The reason could be related to the energy requirements in forming one of the possible intermediates, in that the energy of the transition state going to a particular intermediate may be lower than the others. For example figure **9** shows the three possible intermediates that may form in the first step of the mechanism.

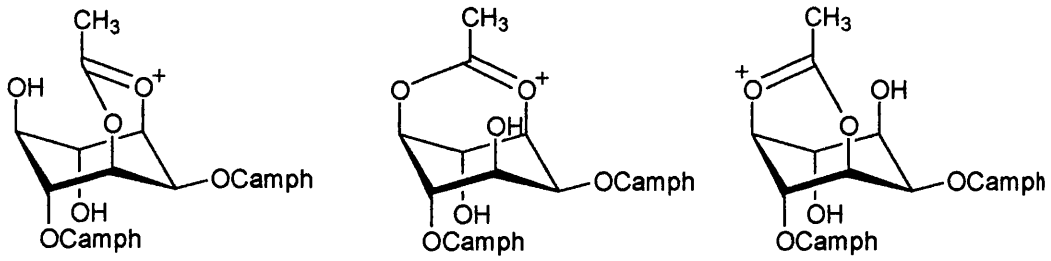
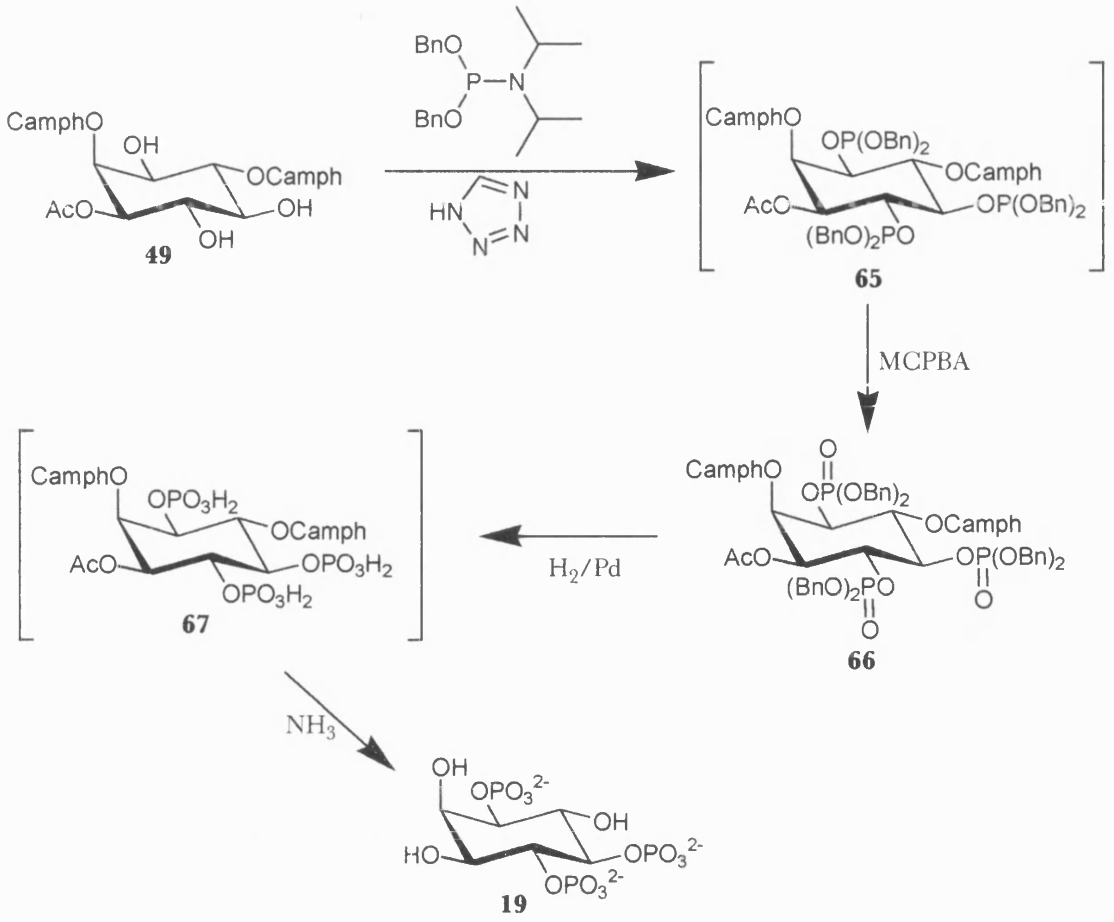


Figure 9 Intermediates during orthoacetate hydrolysis.

Note that formation of both 1- and 3-acetate may result from intermediate **A**, but not the 5-acetate. If the formation of intermediate **A** was favoured over the other two on energetic grounds for example, then no 5-acetate could form during the reaction. It is more feasible that the formation of one intermediate may be favoured over two others, than the protonation of two oxygens being favoured over the third oxygen. The explanation behind the regioselectivity of this reaction is obviously complicated.

4.4 Phosphorylation and Deprotection

Phosphorus-III chemistry was used to introduce the phosphate groups as a phosphorus-V approach may have caused problems such as low reactivity and formation of a five-membered cyclic phosphate at the vicinal 4,5-hydroxyls, as described above. Phosphitylation of **49** (scheme **16**) was carried out using bisbenzyloxydiisopropylamino-phosphine in the presence of 1*H*-tetrazole as catalyst (the phosphitylating reagent-tetrazolide was observed by ^{31}P NMR before committing the triol to the reaction mixture). This intermediate phosphite triester **65** was not isolated but, again, a ^{31}P NMR spectrum (figure **10**) was run on a sample of the reaction mixture to check that reaction with the alcohols had occurred. This is possible as the phosphitylating reagent-tetrazolide is observed at around δ_P 127 and a shift downfield to *ca.* δ_P 140 is observed upon formation of the phosphite triester. The trisphosphite triester was immediately oxidised to the trisphosphate triester using excess MCPBA at −78°C, yielding the dibenzyl protected trisphosphate **66**, after flash chromatography, in 72% yield. ^{31}P NMR chemical shifts show a marked change upon oxidation of phosphorus; figure **10** shows the ^{31}P spectrum of **65**, the trisphosphite triester and of the trisphosphate triester **66**. Note the $^5J_{PP}$ couplings, in the spectrum of **65**, of the 4,5-vicinal phosphites giving rise to doublets. This pattern of couplings, an AB system and one singlet, confirms the regiochemistry of **49**; an acetate at the 1-position would have led to more complex signals upon phosphitylation.



Scheme 16 Phosphorylation and deprotection of **49** yields Ins(1,4,5)P_3

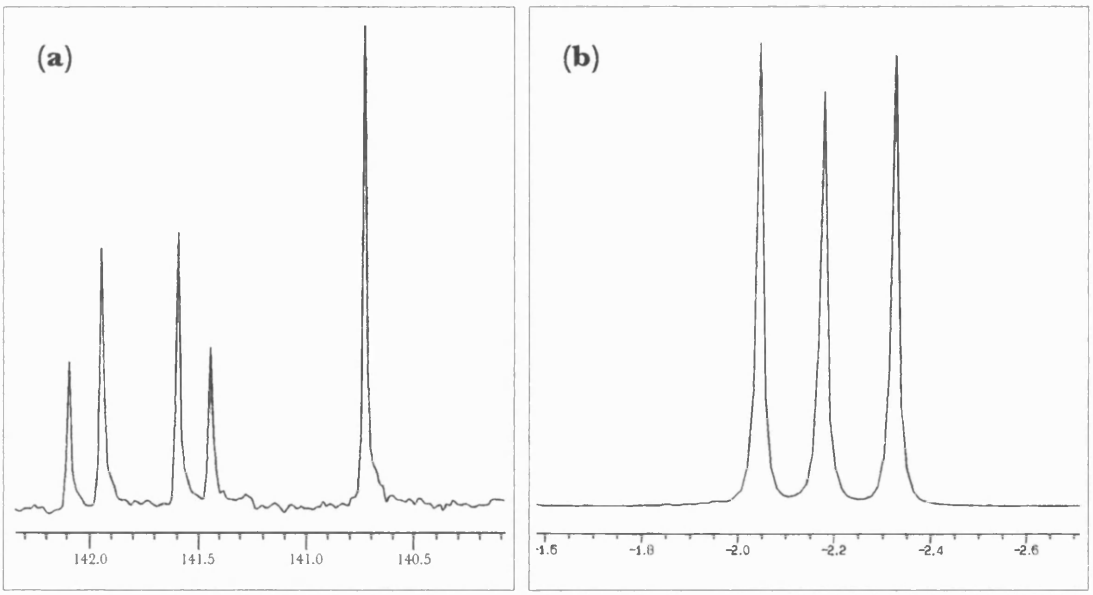


Figure 10 Proton-decoupled ^{31}P NMR spectra of (**a**) trisphosphite triester **65** (90MHz) and (**b**) trisphosphate triester **66** (162MHz).

The benzyl groups were removed by hydrogenolysis over 10% palladium on charcoal at atmospheric pressure; then the camphanates and acetate were hydrolysed with ammonia at 60°C (after removal of the catalyst and solvent) to yield Ins(1,4,5)P_3. Deprotection in this order ensured that the risk of phosphate migration was minimised. Although the only non-volatile impurities at this stage were presumably acetamide and camphanamide, and these could presumably be washed out with a suitable organic solvent, a product of high purity was best obtained after ion exchange chromatography. This also removed any traces of palladium ions which may have been present after the catalytic hydrogenolysis, which may give rise to poor NMR spectra. The Ins(1,4,5)P_3 was purified on a strong anion exchanger, Q Sepharose Fast Flow resin, eluting with a gradient of triethylammonium hydrogencarbonate buffer to yield the triethylammonium salt. As the product contains no chromophore, it was not possible to detect, by UV absorption, which column fractions contain the product. Therefore, the fractions were analysed for phosphate content by a method where the compound is broken down to free phosphate, which is then detected by formation of a blue complex with molybdate ions. This is based upon the assay first described by Briggs [Briggs, 1922]. This assay was then used again, quantitatively, to determine how much Ins(1,4,5)P_3 had been synthesised. This is necessary, as the exact stoichiometry, *i.e.* the number of triethylammonium ions associated with each phosphate group, of the triethylammonium salt cannot be predicted with accuracy; therefore, one may not simply weigh it. For the quantitative assay, a standard curve was prepared by assaying known amounts of potassium dihydrogen phosphate. Thus the amount of phosphate contained in samples of Ins(1,4,5)P_3 could be calculated and, hence, the amount of Ins(1,4,5)P_3 itself. The triethylammonium salt of Ins(1,4,5)P_3 was obtained as a colourless glass in 70% yield, figure **11** shows the ^{1}H NMR and the ^{1}H-decoupled ^{31}P NMR spectra.

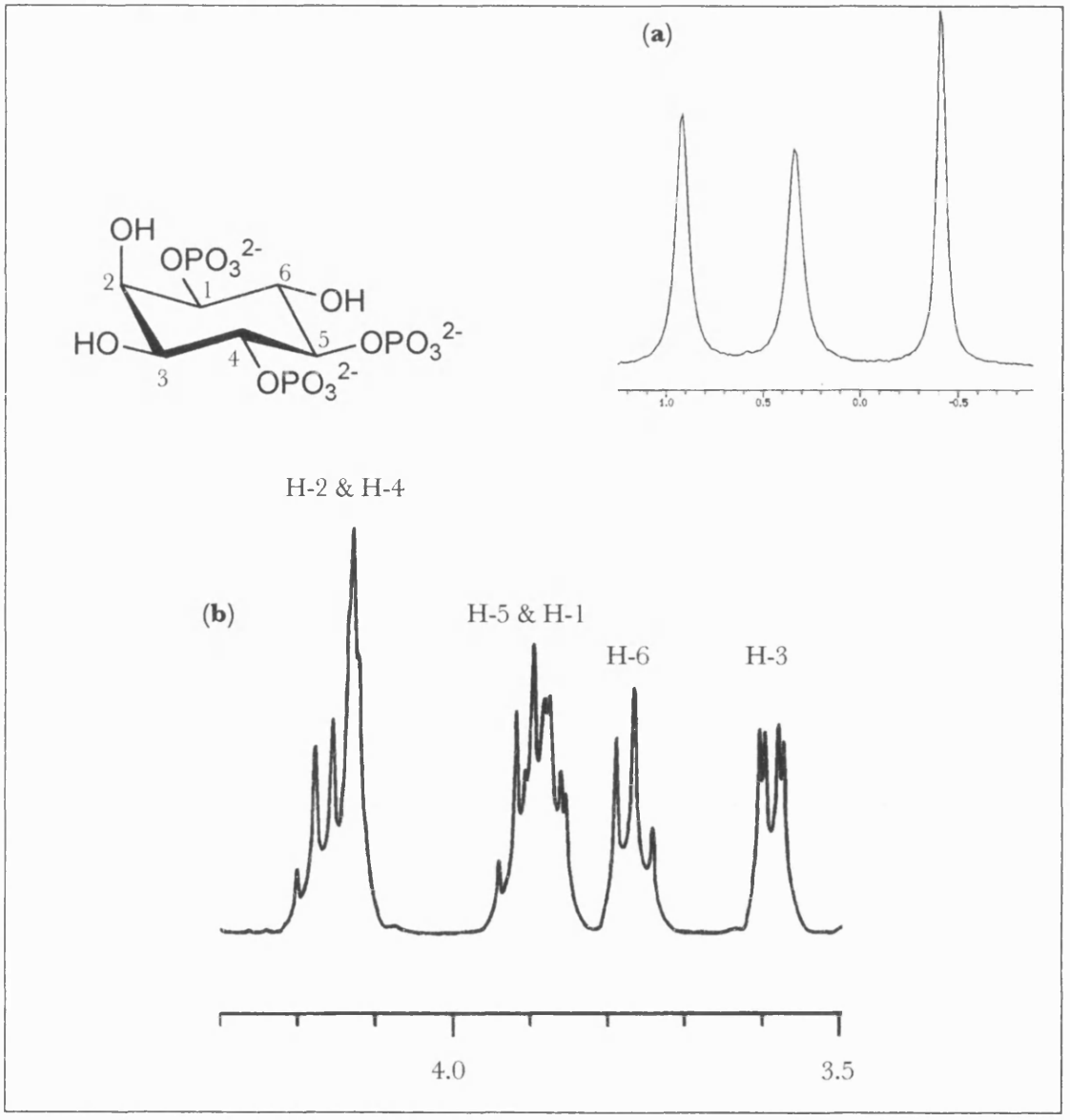


Figure 11 (**a**) ^{31}P NMR (162MHz, D_2O) and (**b**) ^{1}H NMR (400MHz, D_2O) spectra of the triethyl ammonium salt of Ins(1,4,5)P_3.

5 Biological Evaluation

I am grateful to Dr Colin Taylor and co-workers, Department of Pharmacology, University of Cambridge who performed these assays.

The ability of this synthetic Ins(1,4,5)P_3 to stimulate Ca^{2+} release was determined in permeabilised rat hepatocytes. Hepatocytes were isolated from male Wistar rats and permeabilised by incubation with saponin in a cytosol-like medium (CLM). The cells were loaded to steady state with $^{45}Ca^{2+}$ at 37°C in CLM, then further Ca^{2+} uptake was prevented by treatment with thapsigargin. Appropriate concentrations of Ins(1,4,5)P_3 were added and, after

60 seconds, the preparation was quenched in an ice-cold medium. The $^{45}Ca^{2+}$ content of the stores was determined using a rapid filtration method. The dose-response curve is shown in figure **12** and table **3** gives the EC_{50} value.

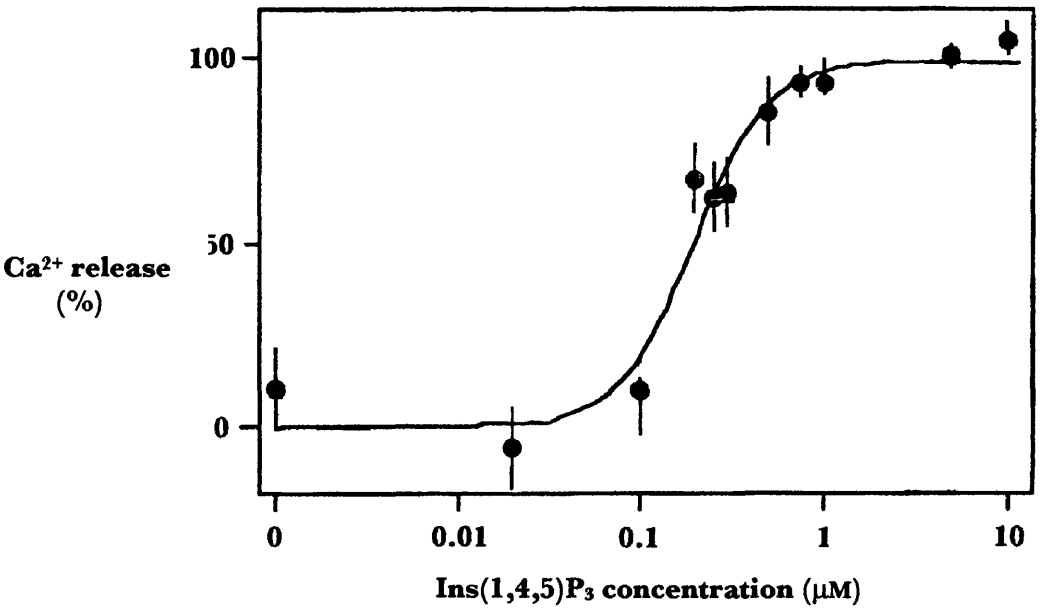


Figure 12 Effect of synthetic Ins(1,4,5)P_3 on Ca^{2+} mobilisation in permeabilised rat hepatocytes

	EC_{50} (nM)
Commercial sample	122 ± 8
Synthetic	121 ± 2

Table 3 EC_{50} values in permeabilised rat hepatocytes for Ins(1,4,5)P_3 made by the route described in this chapter compared to a commercial sample.

6 Conclusions

A rapid, novel route to Ins(1,4,5)P_3 from *myo*-inositol is described. The chemical analyses show this route produces material of high purity and this is confirmed by the biological evaluation where the synthetic material prepared *via* this route is indistinguishable in its intracellular Ca^{2+} release profile from a commercial sample. This route offers considerable advantages over established routes in terms of facility, rapidity and potential for large scale synthesis. Further, the route provides intermediates that may find use in the synthesis of other targets. The 2,4-diester **63** should provide L-Ins(1,4,5)P_3 *via* an analogous route and the 1-acetate ester **60** may find use in the synthesis of other inositol phosphate analogues or phospholipids.

7 Publication

The synthesis of D-*myo*-inositol 1,4,5-trisphosphate described in this chapter was published as the following communication in *Journal of the Chemical Society Perkin Transactions 1*.

Rapid and practical synthesis of D-*myo*-inositol 1,4,5-trisphosphate

COMMUNICATION
PERKIN 1

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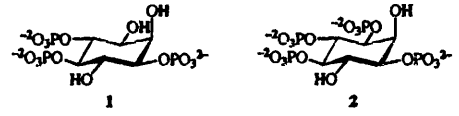
A concise synthetic sequence to biologically active D-*myo*-inositol 1,4,5-trisphosphate is described involving just five steps from *myo*-inositol and minimal chromatography with a key transformation of orthoacetate into acetate protection.

D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3, **1**] is a cellular second messenger first identified in 1983.[1] Phospholipase C catalyses hydrolysis of phosphatidylinositol 4,5-bisphosphate to Ins(1,4,5)P_3 and diacylglycerol. Ins(1,4,5)P_3 interacts specifically with a tetrameric receptor-operated Ca^{2+} channel on the endoplasmic reticulum to mobilise Ca^{2+} stores in stimulated cells.[2] Ins(1,4,5)P_3 mediates the agonist-induced response *via* this rise in intracellular Ca^{2+} concentration. There is continuing interest in the biology of Ins(1,4,5)P_3 and the many other related inositol polyphosphates, and in the synthesis of analogues that may offer the prospect of pharmacological intervention in this signalling pathway.[3]



1 **2**

Structures of D-Ins(1,4,5)P_3 **1** and D-Ins(1,3,4,5)P_4 **2**

Since Ozaki and co-workers first prepared optically active Ins(1,4,5)P_3 in 1986,[4] many routes have been described for the synthesis of enantiomerically pure Ins(1,4,5)P_3 from diverse starting materials.[3] They are generally time-consuming long linear sequences involving extensive chromatography. The critical strategic points in most routes to chiral inositol phosphates are desymmetrisation of *myo*-inositol (a *meso* compound) and resolution of an intermediate. The most rapid route to chiral **1** hitherto described is probably that of Salamonczyk and Pietrusiewicz.[5] This route, however, involves a difficult precipitation-driven equilibrium and is not readily modified to provide materials leading to other inositol phosphate derivatives. Here, we describe a shorter synthetic sequence which obviates the need for tedious chromatographic separations and long sequences of protecting group manipulations and which, by modification of reaction conditions and/or isolation procedures, can provide material for alternative targets. The route is also applicable to relatively large scale preparations.

We have recently published[6] a rapid synthesis of D-*myo*-inositol 1,3,4,5-tetrakisphosphate [D-Ins(1,3,4,5)P_4, **2**] and its enantiomer L-Ins(1,3,4,5)P_4 by a chiral desymmetrisation approach. In acid catalysed deprotection of intermediates utilising orthoformate protection[7] we often observed small quantities of formate esters as products of partial deprotection. While these esters were not stable enough to be useful synthetically, this finding did suggest that a different orthoester such as an orthoacetate might be useful as an intermediate protecting group that could be later transformed by partial hydrolysis into an acetate ester of defined regiochemistry such as **8**‡ (Scheme 1). Building upon the above we have now developed a synthesis of chiral Ins(1,4,5)P_3 involving only five steps from *myo*-inositol. Furthermore, the methodology developed has potential application in the synthesis of other inositol phosphates. *myo*-Inositol orthoacetate **4**§ was prepared by modifying methodology established for the synthesis of the orthoformate orthoester of *myo*-inositol.[8] *myo*-Inositol was treated with triethyl orthoacetate and PTSA in DMF. The acid was removed by precipitation as a salt. After filtration and evaporation *in vacuo* the residue was dissolved in hot methanol and filtered to remove any unreacted *myo*-inositol. Recrystallisation from methanol–chloroform–light petroleum followed by further recrystallisation from methanol gave *myo*-inositol orthoacetate **4** of sufficient purity for use in the next step, in 60–65% yield. Orthoacetate **4** was treated with 2.1 equivalents of 1*S*-(−)-camphanoyl chloride and a catalytic amount of DMAP in dichloromethane to yield the two diastereoisomeric dicamphanates **5**¶ and **6**. In our synthesis of the enantiomers of *myo*-inositol 1,3,4,5-tetrakisphosphate,[6] careful chromatography was required to separate the analogous 2,4- and 2,6-diastereoisomeric dicamphanate esters of *myo*-inositol orthoformate. As a consequence of the orthoacetate functionally, the corresponding diesters **5** and **6** were easier to crystallise and thus were separable without chromatography. Crystallisation from ethyl acetate gave the required D-2,6-diester **5** in 37% yield. Control over the selectivity of the desymmetrisation could be achieved by changing the reaction conditions. An excess

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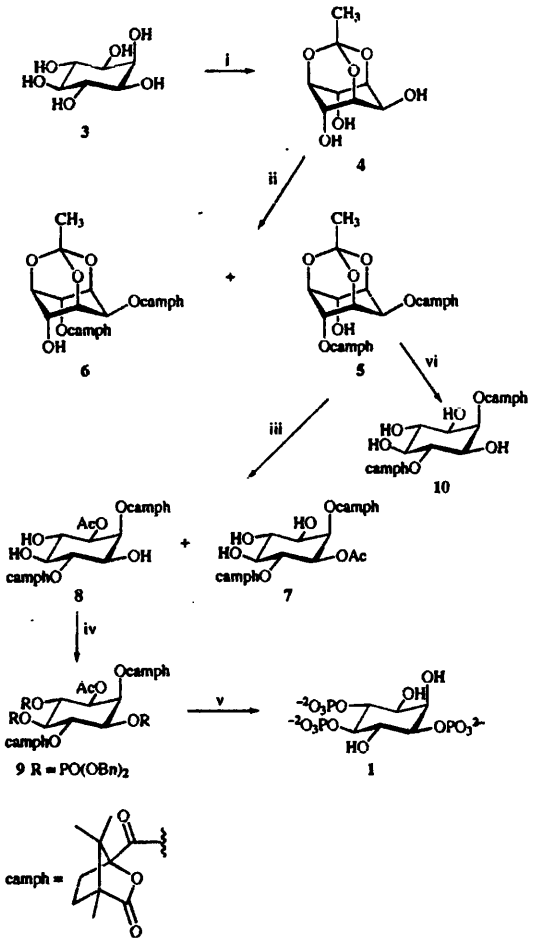
‡ Data for compound **8**: mp 226–229 °C; $[\alpha]_D$ +5.2 (*c* 1.9, DMF) (Found: C, 57.5; H, 6.5. Calc. for $C_{28}H_{38}O_{15}$: C, 57.63; H, 6.74%); δ_H(400 MHz; $[^2H]_7$DMF; TMS) 0.96, 1.02, 1.05, 1.10, 1.12, 1.17 (18H, 6 × s, camph CH_3), 1.55–1.67 (2H, m, camph CH_2), 1.94–2.14 (4H, m, camph CH_2), 2.02 (3H, s, $COCH_3$), 2.49–2.61 (2H, m, camph CH_2), 3.61 (1H, dt, H-5, *J* 5.9, 9.3 Hz D_2O, exchange gives t, *J* 9.8 Hz) 3.86 (1H, dt, H-4, *J* 4.9, 9.8 Hz, D_2O exchange gives t, *J* 9.8 Hz), 4.15–4.17 (1H, m, H-1, D_2O exchange gives dd, *J* 2.8, 10.4 Hz), 4.96 (1H, dd, H-3, *J* 2.8, 10.1 Hz), 5.29 (1H, t, H-6, *J* 10.1 Hz), 5.58 (1H, d, 5-OH, *J* 5.4 Hz), 5.60 (1H, m, 4-OH), 5.62–5.63 (1H, m, H-2, D_2O exchange gives t, *J* 2.8 Hz), 5.75 (1H, d, 1-OH, *J* 5.9 Hz); *m/z* (FAB+) [Found: $(M + H)^+$, 583.2394. $C_{28}H_{39}O_{15}$ requires 583.2391].

§ Data for compound **4**: mp 185–187 °C (with softening at 165 °C) (Found: C, 47.0; H, 6.0. Calc. for $C_8H_{12}O_6$: C, 47.06; H, 5.92%); δ_H(400 MHz; $[^2H]_6$DMSO) 1.28 (3H, s, CH_3), 3.94–4.0 (4H, m, Ins-H), 4.21–4.27 (2H, m, Ins-H), 5.19 (1H, s, 2-OH), 5.38 (2H, d, 4-OH and 6-OH, *J* 5.2 Hz); δ_C(100 MHz; $[^2H]_6$DMSO) 24.30 (CH_3), 57.70, 67.24, 69.24, 74.98 (Ins C), 107.66 (CCH_3); *m/z* (FAB+) 205 [$(M + H)^+$, 100%].

¶ Data for compound **5**: mp 228–231 °C; $[\alpha]_D^{24}$ +17 (*c* = 1, CH_2Cl_2) (Found: C, 59.6; H, 6.45. Calc. for $C_{28}H_{36}O_{12}$: C, 59.59; H, 6.43%); δ_H(400 MHz; $CDCl_3$; TMS) 0.98, 0.99, 1.08, 1.09, 1.10, 1.11 (18H, 6 × s, camph CH_3), 1.44 (3H, s, O_2CCH_3), 1.67–1.76 (2H, m, camph CH_2), 1.91–2.10 (4H, m, camph CH_2), 2.39–2.53 (2H, m, camph CH_2), 3.23 (1H, d, 2-OH, *J* 6.4 Hz), 4.33–4.36 (1H, m, H-3), 4.39–4.43 (1H, m, H-1), 4.45–4.50 (1H, m, H-5), 4.57–4.62 (1H, m, H-4), 5.21–5.25 (1H, m, H-2), 5.52–5.57 (1H, m, H-6); δ_C(100 MHz; $CDCl_3$) 9.59, 9.66, 16.47, 16.54, 16.65 (camph CH_3), 23.94 (O_2CCH_3), 28.76, 28.90, 30.44, 30.86 (camph CH_2), 54.41, 54.51, 54.80, 54.89, 90.86, 91.03 (camph C), 63.72, 66.78, 68.15, 69.14, 69.30, 71.86 (ins C), 108.8 (O_2C), 166.15, 166.81, 177.73, 178.02 (camph CO); *m/z* (FAB+) 565 [$(M + H)^+$, 100%].



Scheme 1 *Reagents and conditions*: i, Triethyl orthoacetate, PTSA, DMF, 90–100 °C, 4 h; ii, 1*S*-(−)-camphanoyl chloride, DMAP, dichloromethane, 0 °C then RT, 2 h; iii, 80% aqueous trifluoroacetic acid, 7 days; iv (*a*), $(BnO)_2PNPr^i_2$, 1*H*-tetrazole, dichloromethane, RT, 1 h; (*b*), MCPBA, dichloromethane, −78 °C to RT; v (*a*), H_2, 10% Pd on C, MeOH–H_2O, RT, 12 h; (*b*), conc. aqueous NH_3, 60 °C, 4 h; vi, 5 M HCl–MeOH 1:11, reflux, 14 h

of the 2,4-diester **6** was gained by omitting the DMAP, and crystallisation from methanol gave **6** in 40–45% yield. The regiochemistry of the required 2,6-dicamphanate **5** was determined by removing the orthoacetate completely by refluxing in methanol–HCl to give tetrol **10**. The $[\alpha]_D$ value of **10** was found to be identical to that of authentic material {$[\alpha]_D^{25}$ −8 (*c* 1, DMF)} synthesised from the analogous 2,6-dicamphanate with orthoformate protection, a compound whose structure had been unambiguously determined previously by X-ray crystallography.[6]

Selective transformation of **5** to **8** was required to produce the appropriately protected intermediate for the synthesis of **1**. Whereas acidic hydrolysis of the analogous orthoformate, as described in our synthesis of **2**, resulted in complete loss of the protection afforded by this functionality, treatment of **5** with 80% aqueous trifluoroacetic acid at room temperature for one week yielded the acetate esters **7** and **8**. Regioisomer **8**, required to make $Ins(1,4,5)P_3$, could be crystallised directly from the mixture in 25% yield using methanol–light petroleum (total yield 31%). Pure **7** could be obtained in 40% yield by chromatography of the mother liquor (R_F **8** 0.32, **7** 0.47, [EtOAc]). Structure determination of these acetate esters was straightforward by ^1H-^1H COSY NMR experiments. No derivative with a 5-acetate was obtained. Phosphitylation of **8** using dibenzyl *N*,*N*-diisopropyl phosphoramidite[9] followed by oxidation of the intermediate phosphite triester with MCPBA gave the protected phosphate **9** in 72% yield. One-pot deprotection by hydrogenolysis at atmospheric pressure over 10% Pd/C, then ester hydrolysis with ammonia at 60 °C gave $Ins(1,4,5)P_3$. Deprotection in this manner ensured that any risk of phosphate migration was minimised. For biological evaluation, the $Ins(1,4,5)P_3$ **1** was further purified by ion exchange chromatography on Q-Sepharose Fast Flow resin eluting with a gradient of triethylammonium hydrogen carbonate buffer (0–100% 1 M) to give the triethylammonium salt in 70% yield (quantified by Briggs phosphate assay[10]). A typical preparation generated 60 mg of this highly active target compound. Clearly the 2,4-diester should provide the now well-established biological control L-$Ins(1,4,5)P_3$[11] *via* an analogous synthetic route. The other readily available regioisomeric acetate **7** could find use in the synthesis of further inositol polyphosphates or phospholipids.

Biological evaluation using saponin-permeabilised hepatocytes showed that synthetic **1** prepared *via* this route was indistinguishable in its intracellular Ca^{2+} release profile (EC_{50} = 121 ± 2 nM) from a sample of commercially available $Ins(1,4,5)P_3$ (EC_{50} = 122 ± 8 nM).

In summary, we have described a novel route to this highly potent second messenger in chiral form with considerable advantages over currently established routes in terms of facility, speed and potential for large scale synthesis.

Acknowledgements

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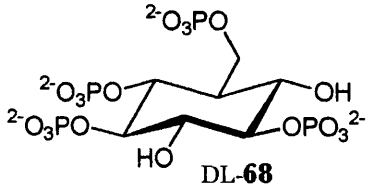
Chapter 3:
Synthesis of an Inositol Tetrakisphosphate Analogue

1 Introduction

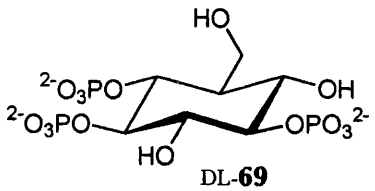
This chapter highlights the importance of D-*myo*-inositol 1,3,4,5-tetrakisphosphate, the product of the action of 3-kinase on Ins(1,4,5)P_3, and describes the synthesis of the tetrakisphosphate **68** in racemic form. The rationale behind the synthesis of the 3-deoxy-*scyllo* inositol derivative DL-**68** was twofold:



DL-**68**

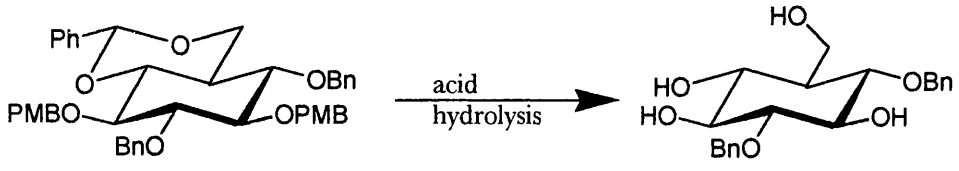
(*i*) There is currently a high level of interest in Ins(1,3,4,5)P_4 and in Ins(1,4,5)P_3 3-kinase. Compound DL-**68** is an interesting Ins(1,3,4,5)P_4 surrogate and could be tested for activity at the Ins(1,4,5)P_3 receptor and against the metabolic enzymes, providing valuable structure-activity data.

(*ii*) The Ins(1,4,5)P_3 surrogate **69** had been synthesised in racemic form [Riley *et al*, 1996; Riley,1996] and had been tested as a substrate for Ins(1,4,5)P_3 3-kinase. The enzyme phosphorylated this molecule; however, it was not certain at which position the new phosphate ester had formed. Phosphorylation of the primary hydroxyl to give D/L-**68** would be an unusual and interesting observation. Synthesis of DL-**68** would provide a standard that could be used in HPLC to determine whether or not D/L-**68** forms as a result of the action of 3-kinase on DL-**69**.



DL-**69**

The fully protected inositol derivative DL-**70** was chosen as the key intermediate for the synthesis of DL-**68**. The synthesis of DL-**70** has been demonstrated in excellent yield starting from *myo*-inositol [Riley *et al*, 1998]. Treatment of DL-**70** with acid should remove the benzylidene acetal and the 4-methoxybenzyl ether protection to yield DL-**71**. Phosphitylation/oxidation then deprotection of DL-**71** would then yield the desired compound DL-**68** (scheme **17**).



DL-**70** DL-**71**

Scheme 17 Acid hydrolysis of fully protected inositol derivative DL-**70** would yield key intermediate DL-**71**.

2 D-*myo*-Inositol 1,4,5-Trisphosphate 3-Kinase

2.1 Introduction

D-*myo*-Inositol 1,4,5-trisphosphate 3-kinase catalyses the phosphorylation of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4. The enzyme was first identified in animal tissues in 1986 [Irvine *et al*, 1986]. Ins(1,3,4,5)P_4 had recently been discovered [Batty *et al*, 1985] and the search was on for the metabolic steps that generated it. It was found that 10-20% of [^{3}H]-Ins(1,4,5)P_3 microinjected into *Xenopus* oocytes was rapidly converted to a tetrakisphosphate and greater conversion was observed with rat tissue homogenates (brain, liver and pancreas). The new tetrakisphosphate was identified as Ins(1,3,4,5)P_4; thus Ins(1,4,5)P_3 3-kinase activity had been demonstrated [Irvine *et al*, 1986].

2.2 There are Different Isoforms of 3-Kinase

Two isoforms of 3-kinase have now been described, referred to as Ins(1,4,5)P_3 3-kinase A and B [Communi *et al*, 1995] and a putative third isoform, Ins(1,4,5)P_3 3-kinase C, has also been suggested. However, care must be taken when identifying and describing different isoforms as there has been the suggestion that, although some reported 3-kinase activity of low molecular weight cytosolic proteins may indeed correspond to different isoforms, some activity is likely to derive from proteolytic† release of the C-terminal region which contains the catalytic domain [Soriano and Banting, 1997]. The C-terminal region shows high amino acid identity between A and B isoforms [Woodring and Garrison, 1997]. There is little identity at the N-terminal region and this region contains many more amino acids in the B isoform. Most studies suggest that the enzymes are largely cytosolic. A study, using Northern analysis to detect mRNA complementary to Ins(1,4,5)P_3 3-kinase cDNA in various rat tissues and human cell cultures, has suggested that Ins(1,4,5)P_3 3-kinase isoforms are expressed in a tissue- or cell-specific manner [Vanweyenberg *et al*, 1995]. In rat tissue, isoenzyme A is specifically expressed in brain and testis, whereas isoenzyme B is predominantly expressed in lung but also in thymus, heart, testis and brain. In the three human cell lines analysed, SH-SY5Y (a neuroblastoma-cell line), HL-60 (a leukaemia-cell line) and HTB-138 (a glioma-cell line), no signal was observed with the isoenzyme A probe, but all three expressed mRNA for isoenzyme B. Rat 3-kinase B has been shown to be associated with the cytosolic face of the ER membrane [Soriano *et al*, 1997] *via* specific, conformation-dependent, protein-protein interactions, with the catalytic domain facing the cytosol.

† Ins(1,4,5)P_3 3-kinases are reported to be highly sensitive to the calcium-activated protease calpain [Lee *et al*, 1990], and degradation products are commonly found in preparations of 3-kinase.

2.3 Regulation of 3-Kinases

Various mechanisms may regulate the activity of 3-kinases. Regulation by Ca^{2+}/calmodulin, by phosphorylation by PKC or PKA or by phosphorylation by a cAMP-dependent protein kinase is known. The 3-kinase from a number of cell types is stimulated by Ca^{2+}/calmodulin to varying degrees dependent upon the cell or tissue type. For example, rat brain isoenzyme A is stimulated 2–3-fold, whereas an uncharacterised human platelet isoenzyme is stimulated 17-fold [Communi *et al*, 1995]. This suggests that regulation of 3-kinases may be cell or tissue specific. However, this cell- or tissue- specific regulation is probably a reflection of tissue-specific expression of different isoforms and that regulation is *isoform-specific*. Further, a recent study [Woodring and Garrison, 1997] showed activation of 3-kinases to be highly dependent upon the purity of the protein and upon the assay conditions, suggesting that comparison of different studies may not be straightforward. There is, however, good evidence for isoform-specific regulation. In this study [Woodring and Garrison, 1997], pure A and B isoforms were produced recombinantly from a mammalian cell line and the regulation of each by Ca^{2+}/calmodulin and by kinases was examined. It was found that each isoform was activated to a different degree by Ca^{2+}/calmodulin. The effect of phosphorylation of the enzyme by PKA and PKC showed marked differences between isoforms. PKA increased the activity of isoform A in the absence of, and to a lesser extent in the presence of, Ca^{2+}/calmodulin; PKC decreased activity both in the presence and absence of Ca^{2+}/calmodulin. Phosphorylation of the B isoform by either of the protein kinases had no effect in the absence of Ca^{2+}/calmodulin, and decreased activity in the presence of Ca^{2+}/calmodulin suggesting that the interaction of the B isoform with Ca^{2+}/calmodulin is affected by its phosphorylation state. The picture regarding the expression and regulation of 3-kinases is obviously complicated and the enzymes are clearly tightly regulated; this suggests that their product, Ins(1,3,4,5)P_4, plays an important role in Ca^{2+} signalling and inositol phosphate metabolism. This is discussed later.

2.4 Structure-Activity Relationships

Recently Choi *et al* examined the interaction of all the possible regioisomers of *myo*-inositol phosphates with 3-kinase [Choi *et al*, 1997]. They examined the ability of all 38 isomers to inhibit phosphorylation of D-[^{3}H]-Ins(1,4,5)P_3 by rat brain 3-kinase (expressed in *E. coli* and purified). Whilst the study showed nothing particularly striking, it did allow them to build up a model for the binding site of the enzyme. Their model is illustrated in figure **13.**

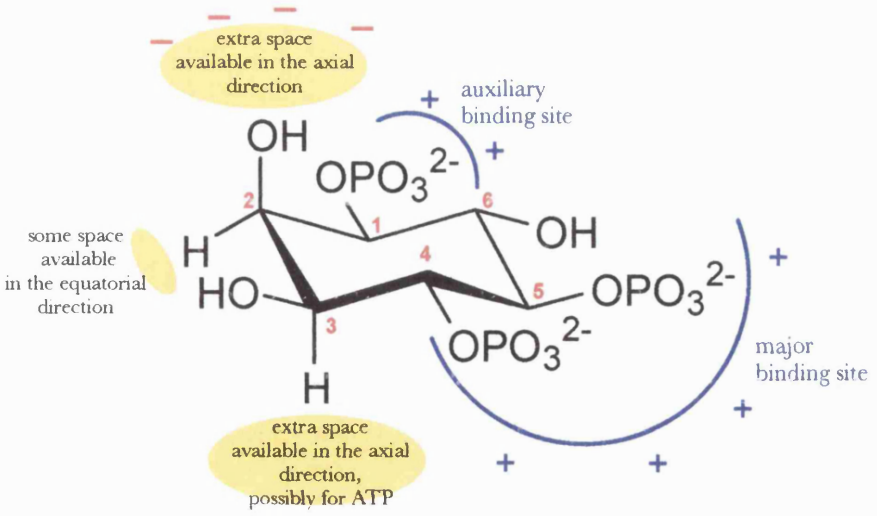


Figure 13 Binding model of Ins(1,4,5)P_3 3-kinase for Ins(1,4,5)P_3 as proposed by Choi *et al* [Choi *et al*, 1997].

Other descriptions of the recognition motifs for this enzyme [for example: Potter and Lampe, 1995] are basically in agreement with the findings of this study. The 1-phosphate seems essential for substrate recognition, both Ins(4,5)P_2 and Ins(2,4,5)P_3 being very poor substrates. The 4- and 5-phosphates are also important for binding, possibly to a slightly lesser extent since Ins(1,4,5)P_3-5S and the equivalent 4,5-bisphosphorothioate are phosphorylated but the trisphosphorothioate is not. Steric bulk at the 2-position is well tolerated, with the exception of phosphate groups as there appears to be negative charge near the vacant space in the axial direction. An interesting 2-position modified analogue is DL-2,2-F_2-Ins(1,4,5)P_3 [Safrany *et al*, 1992]. Upon resolution, the D-isomer is a substrate for 3-kinase, whereas the L-isomer is a potent inhibitor and does not mobilise intracellular Ca^{2+}. Modifications of Ins(1,4,5)P_3 at the 3-position, the site of enzyme action, can result in inhibition. The 3-deoxy and 3-fluoro analogues of Ins(1,4,5)P_3 for example are both inhibitors of 3-kinase. It seems that the enzyme accommodates ATP in the axial direction at the 3-position and that there is limited space in the equatorial direction [Choi *et al*, 1997]. The 6-OH is not important for binding, 6-deoxy-Ins(1,4,5)P_3 is a substrate for 3-kinase but stereoinversion or bulky substituents are not tolerated.

2.5 Inhibitors of 3-Kinases

It can be seen that 3-kinase exhibits considerable stereo and positional selectivity for its substrates and that, in contrast to 5-phosphatase, there has been limited success in the design of selective, potent, non-Ca^{2+} mobilising inhibitors. Probably the best lead to date is the difluoro compound L-2,2-F_2-Ins(1,4,5)P_3, although, interestingly, the cytotoxic anthracycline antibiotic adriamycin appears to be a potent inhibitor of 3-kinase [da Silva *et al*, 1994]. Heparin is also a potent inhibitor of 3-kinase.

3-Kinase works by transferring a phosphate from ATP to Ins(1,4,5)P_3 and, for this, binds both these molecules simultaneously. This could possibly be exploited in the design of a 3-kinase inhibitor. A molecule that binds at both the Ins(1,4,5)P_3 site and the ATP site simultaneously may well be a potent and selective inhibitor of the enzyme, *i.e.* a multisubstrate analogue inhibitor. The ideal multisubstrate analogue would be a conjugate of Ins(1,4,5)P_3 and ATP as shown in figure **14**

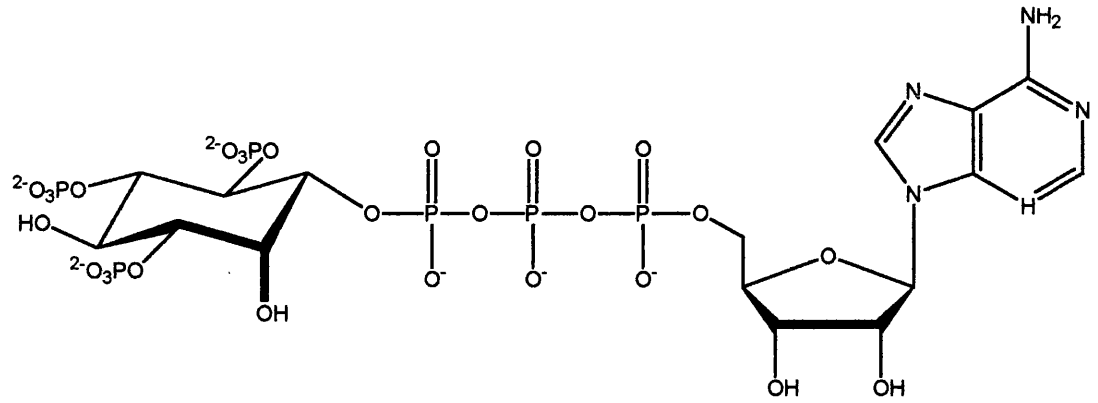
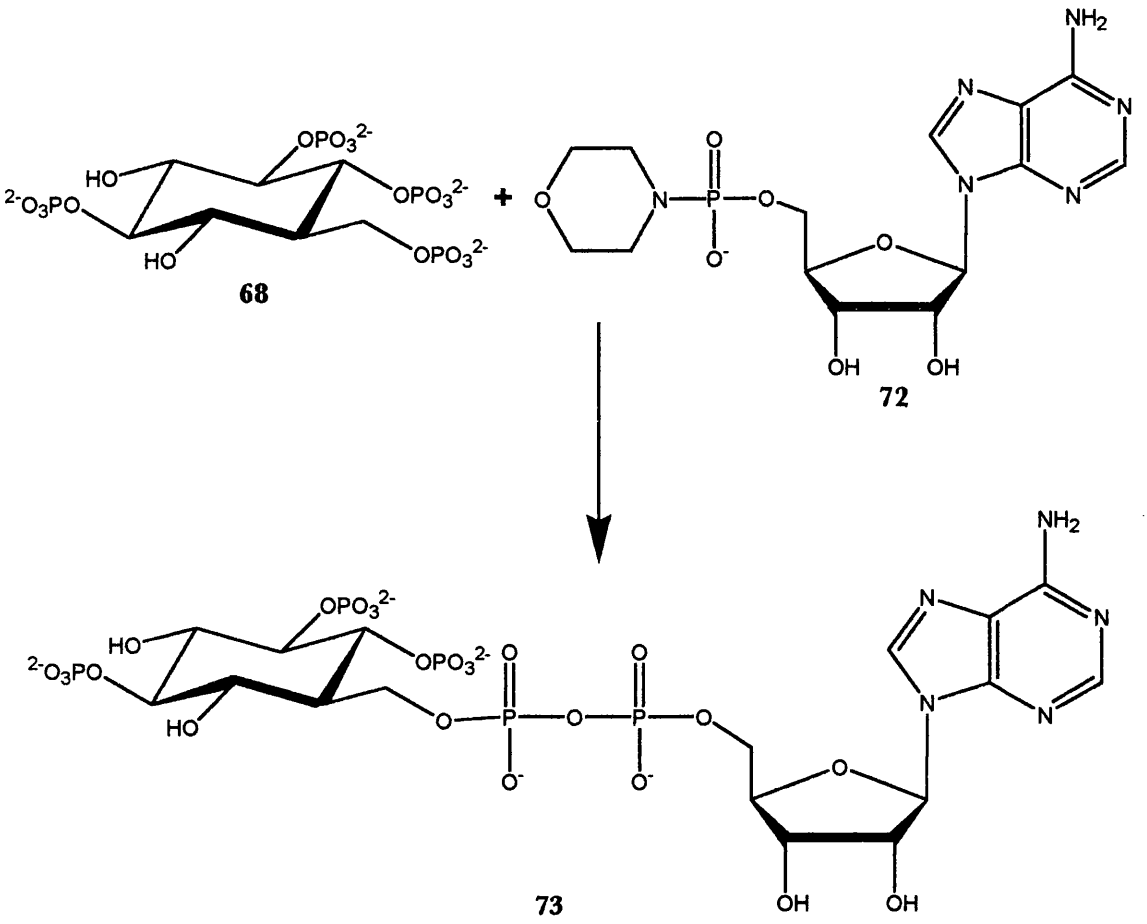


Figure 14 Potentially ideal multisubstrate analogue inhibitor of 3-kinase.

Synthesis of such a molecule would pose many synthetic problems. The coupling of ADP, with an activated diphosphate, to Ins(1,3,4,5)P_4 for example, would be possible but obviously would result in a mixture of regioisomers. Compound **68** may be a useful intermediate in the synthesis of a multisubstrate analogue inhibitor. It is planned to investigate the coupling of **68** to a suitably activated AMP derivative such as its morpholidate (scheme **18**). The conjugate **73** may well be the major, or only product, as the primary phosphate would be expected to be more nucleophilic. Compound **73** differs from the ideal lead compound (figure **14**) only in that it has a methylene in place of one of the phosphates of the triphosphate and would therefore be a useful lead.



Scheme 18 Synthesis of a potential multisubstrate analogue inhibitor of Ins(1,4,5)P_3 3-kinase. The use of morpholidates is well established in making pyrophosphate bonds [for example: Moffatt and Khorana, 1961].

3 Why do Cells Make Ins(1,3,4,5)P_4?

3.1 Introduction

It is now 13 years since the first report of the formation of Ins(1,3,4,5)P_4 in animal tissue, [Batty *et al*, 1985] but the downstream effects of this molecule in cellular signalling remain ambiguous. In particular, two questions remain to be answered;

Can Ins(1,3,4,5)P_4 mobilise Ca^{2+} from intracellular stores by acting at Ins(1,4,5)P_3 receptors?

and

Are there specific receptors for Ins(1,3,4,5)P_4, and, if so, what is their role?

A brief account of the current theories and experimental evidence that addresses these questions will be outlined here.

3.2 Ins(1,3,4,5)P_4 and Ca^{2+} Mobilisation

Studies into whether or not Ins(1,3,4,5)P_4 can mobilise Ca^{2+} by a direct action on Ins(1,4,5)P_3 receptors have yielded some conflicting results, and the picture is further complicated by

potential artifacts. Cells contain a 3-phosphatase enzyme (MIPP); conversion of Ins(1,3,4,5)P_4 to Ins(1,4,5)P_3 is therefore possible. In addition, commercial samples of Ins(1,3,4,5)P_4 have been shown to be contaminated with Ins(1,4,5)P_3. Gawler *et al* [Gawler *et al*, 1990] studied the ability of Ins(1,3,4,5)P_4 to mobilise Ca^{2+} from intracellular stores in saponin-permeabilised SH-SY5Y human neuroblastoma cells. To avoid the considerable conversion of Ins(1,3,4,5)P_4 to Ins(1,4,5)P_3 (*i.e.* 3-phosphatase activity) that they found occurred at room temperature, they performed their experiments at 4°C, which arrests this metabolism. They also found that the activity of commercial samples of Ins(1,3,4,5)P_4 was reduced if pre-treated with a crude Ins(1,4,5)P_3 3-kinase preparation, suggesting contamination with Ins(1,4,5)P_3. They therefore used chemically synthesised Ins(1,3,4,5)P_4. The study showed that Ins(1,3,4,5)P_4 releases Ca^{2+} from intracellular stores and that Ins(1,3,4,5)P_4 appears to act synergistically with sub-maximal concentrations of Ins(1,4,5)P_3 in releasing Ca^{2+}.

Cullen *et al* [Cullen *et al*, 1990], also suggested a synergistic effect between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in Ca^{2+} release, based on a study using digitonin-permeabilised L1210 mouse lymphoma cells. They showed that, in their system, Ins(1,3,4,5)P_4 alone caused no mobilisation of Ca^{2+} but that Ins(1,3,4,5)P_4 markedly enhanced Ins(2,4,5)P_3 induced Ca^{2+} release when Ins(2,4,5)P_3 was administered at low concentration (at threshold or just above). At high Ins(2,4,5)P_3 concentration, Ins(1,3,4,5)P_4 had no effect. In a later study, the same group showed that the dose-response curve to Ins(1,3,4,5)P_4 was not shifted to the left by thimerosal, a sensitiser of the Ins(1,4,5)P_3 receptor [Loomis-Husselbee *et al*, 1996]. This suggests that the synergistic effect of Ins(1,3,4,5)P_4 on Ins(2,4,5)P_3-induced Ca^{2+} release does not appear to involve a direct interaction with the Ins(1,4,5)P_3 receptor but is more likely to be due to an indirect action of Ins(1,3,4,5)P_4 *via* an Ins(1,3,4,5)P_4 receptor.

In contrast, Wilcox *et al* [Wilcox *et al*, 1993a,b] suggested that Ins(1,3,4,5)P_4 independently mobilises Ca^{2+} *via* a direct action on Ins(1,4,5)P_3 receptors. Their studies, using saponin-permeabilised SH-SY5Y cells, showed that Ins(1,3,4,5)P_4 acted as a full agonist, albeit weaker than Ins(1,4,5)P_3 (EC_{50} 2.5μM compared to 52nM). The studies were either conducted in $InsP_6$-supplemented buffer to inhibit 3-phosphatase or with the 3-phosphorothioate analogue of Ins(1,3,4,5)P_4, which is resistant to 3-phosphatase and only a 2-fold weaker agonist. The picture is certainly made no clearer by a more recent study [Bird and Putney, 1996] which has suggested a possible role for Ins(1,3,4,5)P_4 as an inhibitor of the effects of Ins(1,4,5)P_3. The study, using microinjection into mouse lachrymal acinar cells, showed that Ins(1,3,4,5)P_4 was able to antagonise the effects of Ins(2,4,5)P_3 and that it neither mobilised Ca^{2+} nor potentiated the effect of Ins(2,4,5)P_3. They suggest that the contrast shown between their study and the findings of Wilcox *et al* [Wilcox *et al*, 1993a,b] may be explained by the two cell types expressing different forms of the Ins(1,4,5)P_3 receptor.

3.3 Ins(1,3,4,5)P_4 Binding Proteins

Suggestions of a specific Ins(1,3,4,5)P_4 receptor have evolved from the first report of a binding site for Ins(1,3,4,5)P_4 [Bradford and Irvine, 1987]. Binding proteins for Ins(1,3,4,5)P_4 have now been reported in several tissues [Irvine, 1992; Cullen *et al*, 1997]. Cullen *et al* purified and characterised such a protein from pig platelets [Cullen *et al*, 1995b] and studied the binding affinities of the 6 enantiomeric pairs and 3 *meso*-isomers of the regioisomers of Ins(1,3,4,5)P_4 [Cullen *et al*, 1995a] The study concluded that phosphorylation of the 1-,3- and 5-positions is essential for high affinity binding, a 6-phosphate is tolerated but a 2-phosphate is not and a 4-phosphate has very little influence. The protein was later cloned and shown to be a GTPase-activating protein (GAP), specifically, a member of the GAP1 family [Cullen *et al*, 1995c]. They designated the protein GAP1^{IP4BP}.

A pleckstrin homology (PH) domain constitutes part of the GAP1^{IP4BP} protein [Cullen *et al*, 1997]. PH domains are a family of modular domains consisting of sequences of around 100 amino acids originally found in pleckstrin, a major substrate for protein kinase C in platelets. PH domains have been described over 60 different proteins including many involved in cellular signalling, such as the phospholipase C family, various protein kinases and G protein regulators [Shaw, 1996]. Their function appears to be the regulation of membrane binding of various proteins. It is the PH domain of GAP1^{IP4BP} that is the functional Ins(1,3,4,5)P_4 binding site, deletion mutants of GAP1^{IP4BP} that do not contain the PH domain do not bind Ins(1,3,4,5)P_4 [Cullen *et al*, 1997]. Moreover it is the PH domain that appears to be important in determining the localisation of the GAP within the cell. The GAP1^{IP4BP} homologue (about 60% identical), GAP1^{m} shows a different subcellular distribution to GAP1^{IP4BP} in that it appears to have a perinuclear localisation and possibly a small cytosolic component [Lockyer *et al*, 1997]. In contrast, GAP1^{IP4BP} is located solely at the plasma membrane. The difference may be a function of their respective PH domains, as previously noted, the PH domain of GAP1^{IP4BP} contains the Ins(1,3,4,5)P_4 binding site, but also mediates association with the plasma membrane. This suggests that the localisation and function of GAP1^{IP4BP} may be regulated by the competition between phospholipid binding and Ins(1,3,4,5)P_4 binding [Lockyer *et al*, 1997].

Another known binding site for Ins(1,3,4,5)P_4 is the PH domain of Bruton's tyrosine kinase. Mutational changes in this enzyme are the cause of X-linked agammaglobulinaemia a condition in which the function of B cells is disrupted, causing immunodeficiency. Recently, the crystal structure of the PH domain of Bruton's tyrosine kinase in complex with Ins(1,3,4,5)P_4 has been published [Baraldi *et al*, 1999]. This study has provided useful information about the molecular interactions of Ins(1,3,4,5)P_4 and one of its important binding sites. Key interactions involve hydrogen bonds between the 3-, 4- and 5-phosphates and lysine residues and an arginine residue. Ins(1,4,5)P_3 has considerable lower affinity for this site, it is suggested this is due to the

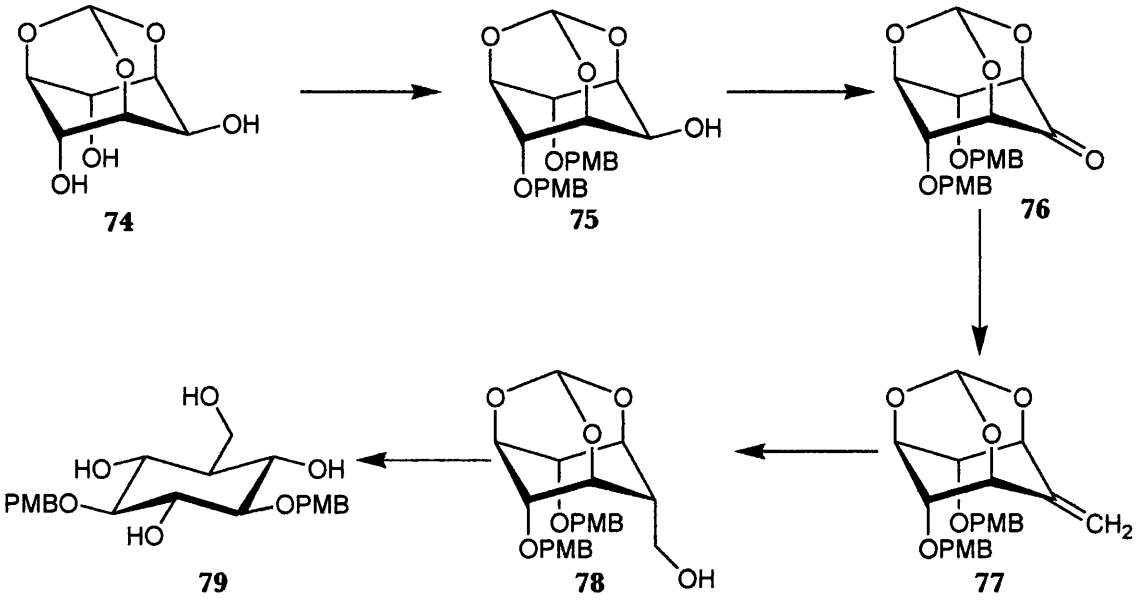
absence of the 3-phosphate significantly reducing the number of hydrogen bonds. The interaction of Ins(1,3,4,5)P_4 directly with the PH domain involves a total of 18 hydrogen bonds. There are further hydrogen bonds *via* water molecules and hydrophobic contacts with the cyclohexane ring.

Although a number of proteins that bind Ins(1,3,4,5)$_4$ with high affinity and specificity have been described, the role of these proteins [Ins(1,3,4,5)P_4 receptors?] in signal transduction is not known. It has been suggested for some years that a possible function of Ins(1,3,4,5)P_4 is to modulate Ca^{2+} entry across the plasma membrane [Irvine, 1992]. Hence, it would seem reasonable that, if GAP1^{IP4BP} is indeed an Ins(1,3,4,5)P_4 receptor, that, given its membrane localisation, it may be involved in this process. In support of the Ca^{2+} entry hypothesis is the identification of an Ins(1,3,4,5)P_4 sensitive Ca^{2+} channel in the plasma membrane of endothelial cells [Lückhoff and Clapham, 1992]. Another study, using adriamycin to inhibit 3-kinase (and therefore Ins(1,3,4,5)P_4 production) in Jurkat T-lymphocytes, showed that the markedly reduced Ins(1,3,4,5)P_4 concentration in the cells did not appear to have an effect on Ca^{2+} entry [da Silva *et al*, 1994]. More attention now focuses upon the physiological targets of GAP1^{IP4BP}, specifically which G-proteins are targets. Both Ras and Rap GAP activity for GAP1^{IP4BP} have been clearly demonstrated *in vitro* [Bottomley *et al*, 1998]. GAP1^{IP4BP} and GAP1^{m} function as GAPs on various Ras family members, when assayed in the presence of membrane phospholipids. The GAP activity is completely dependent on the presence of Ins(1,3,4,5)P_4 suggesting that GAP1^{IP4BP} and GAP1^{m} function as Ins(1,3,4,5)P_4-dependent Ras GAPs. GAP1^{IP4BP} but not GAP1^{m} functions as a Rap GAP and appears not to be regulated by Ins(1,3,4,5)P_4. Thus GAP1^{IP4BP} and GAP1^{m} appear to differ in their specificity as well as their sub-cellular localisation.

4 Synthesis of DL-(1,3,5/2,4,6)-6-Hydroxymethylcyclohexane-1,2,3,4,5-pentol-1,2,4,7-Tetrakisphosphate

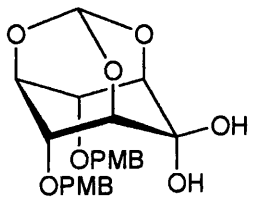
This route starts with the synthesis of *myo*-inositol orthoformate **74** (scheme **19**), using the method of Baudin *et al* [Baudin *et al*, 1988]. The orthoacetate orthoester utilised earlier, in the synthesis of Ins(1,4,5)P_3, is not suitable for use here, as there is a requirement later in the synthesis to remove the orthoester in the presence of 4-methoxybenzyl ethers, which would probably be lost under the conditions required to cleave the orthoacetate. Other protecting groups could be used that would be compatible with the orthoacetate but the advantage of using the PMB group is that its instability to acid should allow the conversion of the fully protected intermediate DL-**70** into the tetrol DL-**71** (scheme **17**). Thus *myo*-inositol was treated

with triethyl orthoformate and PTSA in DMF at 140°C; chromatography and recrystallisation gave the orthoformate **74** as colourless crystals.



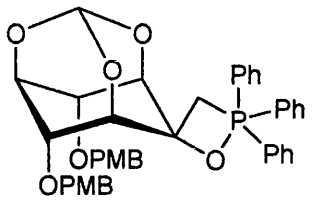
Scheme 19 Synthesis of tetrol **79**.

The route to the fully protected intermediate DL-**70** is that described by Riley *et al* [Riley *et al*, 1998]. Treatment of the orthoformate **74** with 2 equivalents of NaH, then with 2 equivalents of 4-methoxybenzyl chloride in DMF at room temperature gave a mixture of di, mono, and tri-PMB derivatives. Chromatography of the mixture gave the required 4,6-di-PMB derivative **75** in 40% yield. A mild and efficient oxidation of **75** was carried out using a Swern procedure. Anhydrous dimethyl sulfoxide (DMSO), in dichloromethane, was activated using oxalyl chloride at −60°C. This reacts rapidly with the alcohol to form an alkoxysulfonium intermediate that is rapidly converted to the ketone upon addition of triethylamine. Examination of the IR spectrum of the reaction product showed hydroxyl groups at around 3450cm^{-1} as well as a characteristic carbonyl peak at 1760cm^{-1}, indicating formation of the ketone hydrate **80** during the aqueous work up. Boiling a solution of this *gem*-diol in toluene using a Dean and Stark apparatus for azeotropic removal of water, followed by careful recrystallisation using dry solvents under nitrogen, gave the pure ketone **76** in 90% yield. The ease with which the ketone hydrates is attributed to strain effects in this rigid molecule, [Riley *et al*, 1998] the carbon at position-1 preferring to be sp^3-hybridised. A cyclohexane ring can accommodate the larger bond angle at an sp^2 carbon by a small amount of ring distortion, this is obviously not possible in this caged, adamantane-like structure. Alkene **77** was prepared using a Wittig reaction. A solution of potassium *tert*-butoxide in THF was added to a suspension of methyltriphenylphosphonium bromide in THF at 0°C, **76**

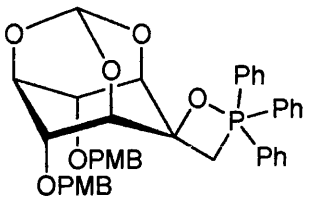


80

was added and the mixture was to be heated to reflux for three hours. The oxaphosphatane intermediate in Wittig reactions of this type is usually unstable, decomposing to the desired alkene at low temperature. The oxaphosphatane of **76**, which presumably has the structure **81a** or **81b** (figure **15**), however, was unusually stable and needed to be heated in refluxing THF in order to decompose. Alkene **77** was purified by column chromatography and was obtained in 84% yield.



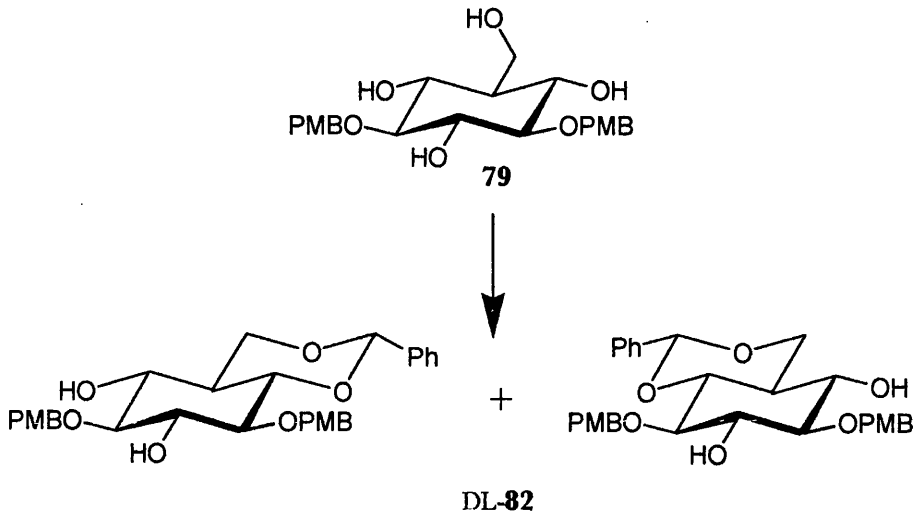
81a



81b

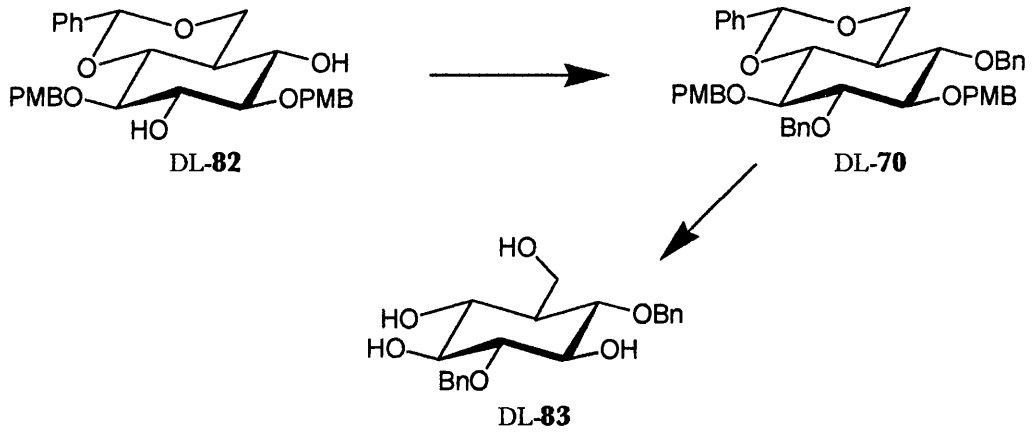
Figure 15 Possible structures of the oxaphosphatane intermediate derived from compound **76.**

Hydroboration/oxidation of **77** using 9-borabicyclononane (9-BBN), then alkaline hydrogen peroxide followed by purification by column chromatography, gave exclusively the axial alcohol **78** in 74% yield. This product is preferred as formation of the equatorial alcohol would require approach by the bulky 9-BBN on the face of the alkenic double bond hindered by the PMB groups. The orthoformate functionality of **78** was hydrolysed by treatment with methanol/1M aqueous HCl at 50°C. The reaction was followed carefully by TLC as the PMB groups will eventually be cleaved under these conditions, so the reaction must be stopped when orthoformate hydrolysis is complete. Treatment of the crude product with concentrated aqueous ammonia was necessary to cleave a formate ester that formed by incomplete orthoformate hydrolysis and was observed by TLC. Chromatography gave the tetrol **79** in 74% yield. Note that each compound synthesised to this stage is *meso*. The next step was to protect the primary hydroxyl and its adjacent secondary hydroxyl simultaneously by forming a benzylidene acetal. It can be seen that this reaction gives a pair of enantiomers, DL-**82** (scheme **20**), therefore each subsequent transformation from this point in the synthesis onwards produces racemic material.



Scheme 20 Treatment of **79** with benzaldehyde dimethyl acetal gives a pair of enantiomers.

Acetal DL-**82** was made in 82% yield by treatment of **79** with benzaldehyde dimethyl acetal in DMF and a catalytic amount of PTSA at 70°C. The reaction vessel was fitted with an air condenser connected to a water pump to remove methanol formed during the reaction; this shortens the reaction time and improves the yield. The remaining two free hydroxyls were benzylated in the usual manner (NaH/BnBr in DMF) to give DL-**70** in 90% yield (scheme **21**). The benzylidene acetal and the PMB groups were removed conveniently in one pot by acidic hydrolysis. Thus treatment with refluxing ethanol/ 1M aqueous HCl (1:20) gave the tetrol DL-**83** in 71% yield, after chromatography.



Scheme 21 Benzylation of racemic **82** followed by acid hydrolysis gave the key intermediate DL-**83**.

Figure **16** shows sections of the ^{1}H NMR spectrum of DL-**83**; a ^{1}H-^{1}H COSY spectrum was required to assign the inositol ring protons.

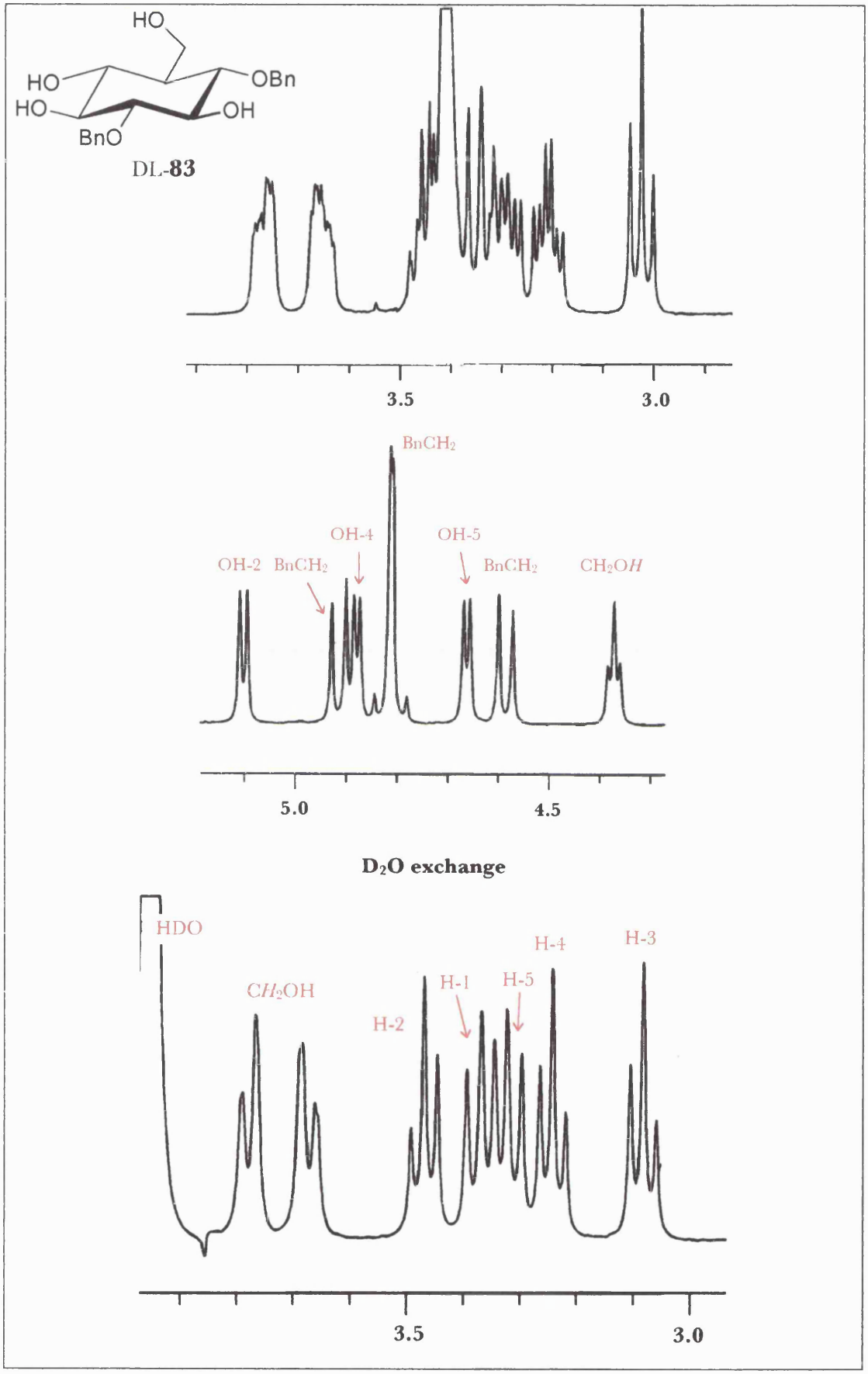
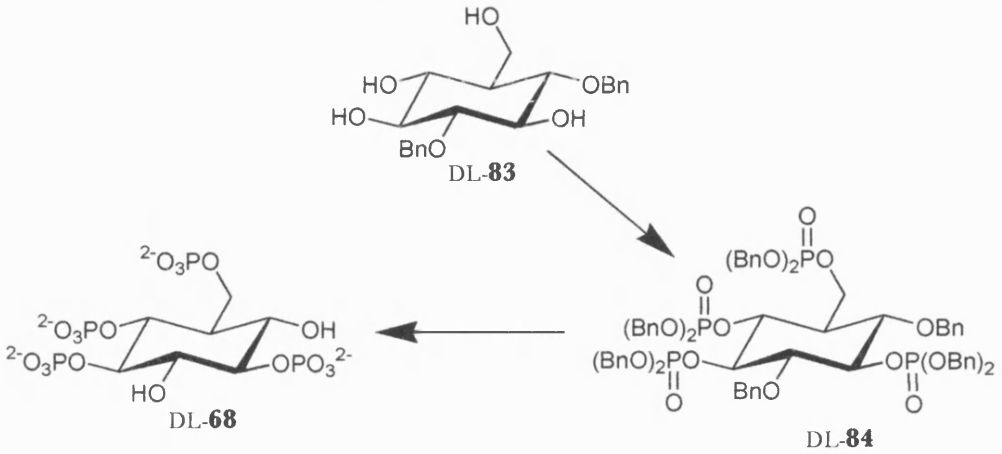


Figure 16 Sections of the ^{1}H NMR spectrum of DL-**83** (400MHz, $[^{2}H]_{6}$-DMSO).

Phosphitylation of DL-**83** with bis(benzyloxy)diisopropylaminophosphine (scheme **22**) in the presence of 1*H*-tetrazole in dichloromethane gave a tetrakisphosphite. This was not isolated but the reaction mixture was checked by ^{31}P NMR (90MHz) and signals were observed at δ140.8 (doublet), 142.3 (singlet), 142.5 (doublet), and 143.0 (singlet). Immediate oxidation with excess MCPBA at −78°C, then purification by column chromatography, gave the benzyl protected tetrakisphosphate DL-**84** in 72% yield.



Scheme 22 Phosphitylation, oxidation, then deprotection of DL-**83** to yield the target tetrakisphosphate DL-**68**.

Deprotection was achieved by catalytic hydrogenolysis using palladium on charcoal under 40psi of hydrogen in MeOH/water (4:1) to yield the tetrakisphosphate DL-**68**. This was purified by ion-exchange chromatography on Q Sepharose Fast Flow resin eluting with a gradient of triethylammonium hydrogencarbonate buffer to yield its triethylammonium salt, 75% as quantified by phosphate assay. Figure **17** shows the ^{31}P NMR spectrum of the final purified product.

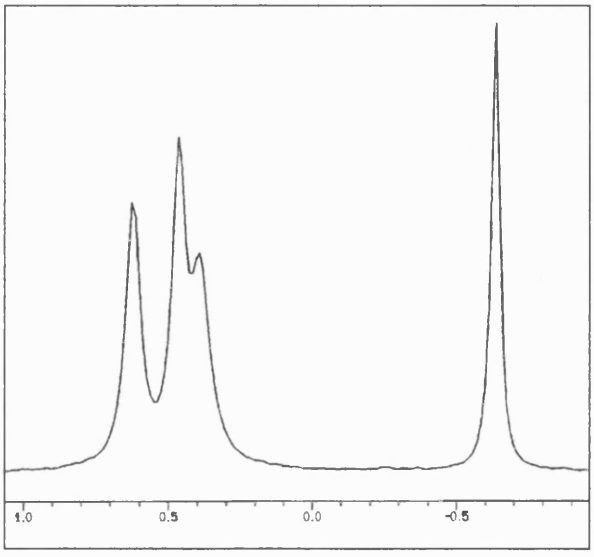


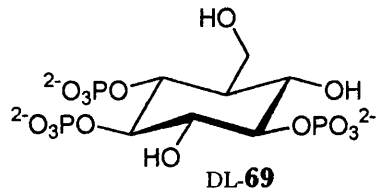
Figure 17 ^{1}H-decoupled ^{31}P NMR spectrum of the tetrakisphosphate DL-**68**

5 Biological Work

5.1 Ca^{2+} Release

I am grateful to Dr Colin Taylor and co-workers, Department of Pharmacology, University of Cambridge who performed the Ca^{2+} release assay.

The ability of compound DL-**68** to stimulate Ca^{2+} release was determined in permeabilised rat hepatocytes using the assay conditions described in chapter 2. *It was found to be inactive.* The 3-hydroxyl of Ins(1,4,5)P_3 is thought to enhance binding to the receptor, probably *via* H-bond interactions. Compound DL-**69** was found to be equipotent with Ins(1,4,5)P_3 itself in releasing $^{45}Ca^{2+}$ from permeabilised rabbit platelets [Riley *et al*, 1996b]. Thus the receptor tolerates the equatorial CH_2OH at this *pseudo* 3-position. Furthermore, this arrangement may even enhance activity, since the compound tested was racemic and presumably only one enantiomer was responsible for the observed activity. Considering DL-**68** as an Ins(1,4,5)P_3 surrogate, the result obtained for Ca^{2+} release indicates that the steric bulk of a phosphate attached to the CH_2OH is not tolerated by the receptor. From this study, little can be concluded when considering DL-**68** as an Ins(1,3,4,5)P_4 mimic. If Ins(1,3,4,5)P_4 does cause Ca^{2+} release, then this result would suggest that the change from secondary to primary phosphate at position 3 is not tolerated (although DL-**68** also differs from Ins(1,3,4,5)P_4 in terms of the 2-hydroxyl being equatorial as opposed to axial, the 2-hydroxyl is not important for Ca^{2+} release).



DL-**69**

5.2 Use as a Standard in Studies Involving 3-Kinase

These studies were performed by G. Mayr and co-workers, University of Hamburg.

As described early in this chapter, DL-**69** had been tested as a substrate for 3-kinase. A product with a retention time in HPLC corresponding to a tetrakisphosphate, or possibly a pentakisphosphate, was observed. The chromatogram‡ in figure **18** shows that the major phosphorylation product that is obtained has a longer retention time than DL-**68**. A small peak does appear to correspond to DL-**68**, this is indicated by the red arrow and suggests that DL-**68** may form as a minor product. Figure **19** illustrates the possible products of phosphorylation of DL-**69**; both tetrakisphosphates and pentakisphosphates may have formed in this experiment. If

‡ The chromatography utilises a metal-dye detection system [Mayr, 1988] as the inositol phosphates possess no chromophore. This is based on the complexation of transition metal ions with both the inositol phosphates and a dye, 4-(2-pyridylazo)resorcinol, at the same time in a so-called "two-ligand one-metal" system. The dye acts as a reporter substance allowing spectrophotometric detection at 546nm.

the enzyme has, indeed, phosphorylated the primary hydroxyl and possibly phosphorylated the molecule twice, this is anomalous to its apparent high substrate specificity discussed earlier in terms of the lack of success with the design and synthesis of inhibitors of 3-kinase.

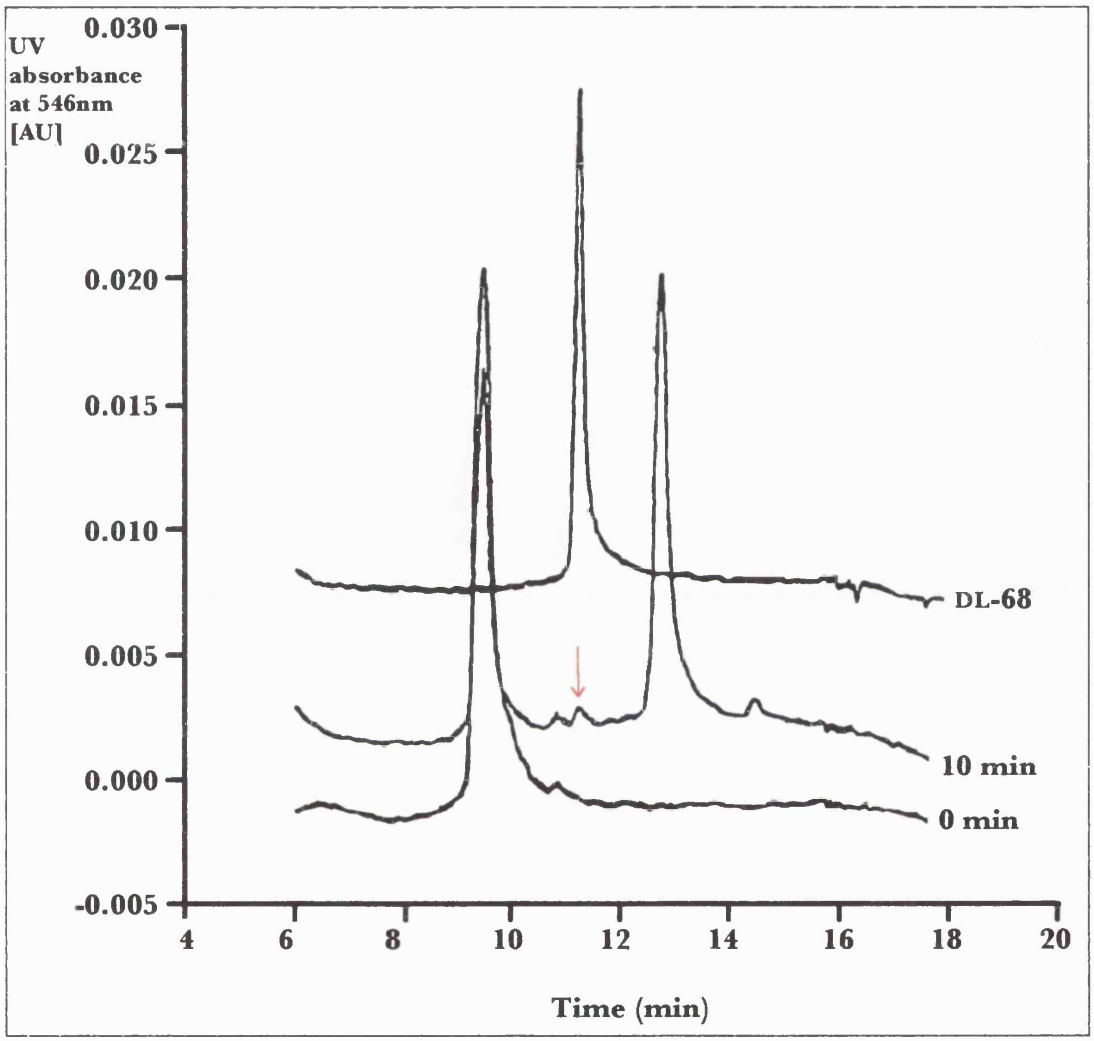


Figure 18 HPLC trace of phosphorylation products of DL-**69** with DL-**68** as a standard.

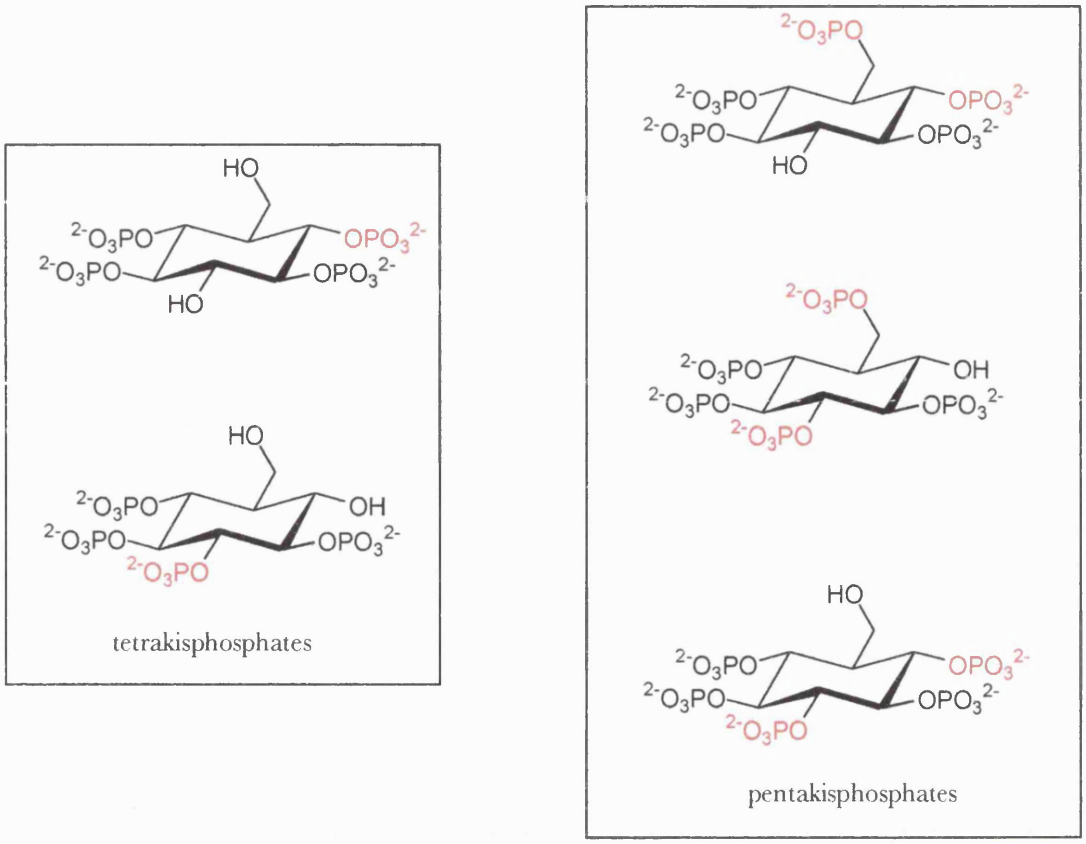


Figure 19 Possible phosphorylation products of DL-**69** other than DL-**68**, new phosphates are shown in red.

As described earlier, the possible phosphorylation of a primary hydroxyl by 3-kinase is a significant and highly interesting result and further experiments were performed in an attempt to gain further information about the phosphorylation of DL-**69**. Figure **20** illustrates the result of an experiment using a lower 3-kinase concentration and a lower ATP concentration.

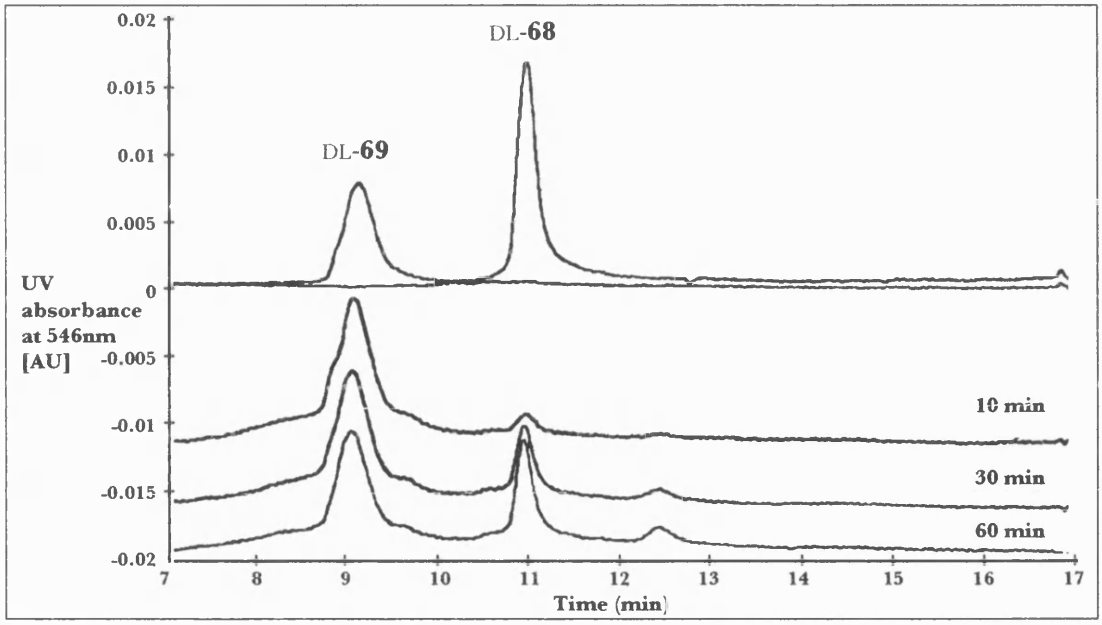


Figure 20 HPLC traces of phosphorylation products of DL-**69** at various timepoints showing formation of DL-**68**.

These data suggest that DL-**68** does form by the action of 3-kinase on DL-**69**. However, this appears to be dependent upon concentration of enzyme and ATP, as a different product, either a pentakisphosphate or a different tetrakisphosphate, forms at higher ATP concentration.

6 Conclusions

The synthesis of a novel inositol tetrakisphosphate is described. Although inactive at the Ins(1,4,5)P_3 receptor, this does make a small contribution to our understanding of the structure-activity relationships of the receptor. The molecule may have use as a probe for the various Ins(1,3,4,5)P_4 binding sites. The molecule has proved useful in a standard in the studies involving 3-kinase and may prove to be useful in the synthesis of a lead compound as a multisubstrate analogue inhibitor of 3-kinase.

PART II:

SYNTHESIS OF mPEG-POLYAMINE CONJUGATES

We can even be somewhat optimistic on the long-range possibility of therapy by the isolation or design, synthesis, and introduction of new genes into defective cells of particular organs.

[Tatum, 1966]

The idea that human genetic disease and even some degenerative and infectious disease will become amenable to correction at the genetic level has cleared its initial conceptual and technical obstacles and has now become widely accepted in most molecular genetic, medical and public policy circles. The first phase of human gene therapy – the emergence and acceptance of the general concept – is over. We are now in an explosive second phase – one of technical implementation.

[Friedmann, 1992]

Nitrogen is the element of faction neatly dividing organic chemists into camps. Those who have mastered its vagaries are undaunted by the prospect of contending with its reactivity. Then there are those in the minority antipodal camp who suspect that nitrogen is best confined to a cylinder and used to shield precious reactions and reagents from the ravages of air and moisture.

[Kocienski, 1994]

Chapter 4:

An Introduction to the Science of Gene Therapy

1 Introduction

The second part of this thesis is concerned with the synthesis of poly(ethylene glycol)-polyamine conjugates. It is thought that such constructs may have use in the development of formulations for the delivery of nucleic acids to cells *in vivo*. Such formulations would have application within the field of **gene therapy**. This chapter will present an introduction to the science behind the well-established concept of gene therapy and highlight the fact that the major obstacle to success in this field is largely a technical one, that of delivering the gene to its site of action, the nuclei of the target cells. A brief overview of how gene therapy has evolved and which diseases it may tackle will be presented, followed by consideration of more technical issues, including the routes by which gene medicines may be administered and what vectors or techniques are available to mediate this delivery.

2 Gene Therapy, an Overview

2.1 What is Gene Therapy?

There are a number of diseases known to be caused by a defect in a specific gene; these conditions are termed monogenic diseases. Such diseases have an obvious cure. Identify the defective gene, produce a functional copy that will produce the correct protein and then administer it to the patient for expression in the required tissue. Thus the synthesis of the missing/defective protein is restored and the genetic defect is corrected. This is the original concept of gene therapy. As research progressed and techniques such as gene sequencing and PCR became commonplace, and much was learned about the molecular basis of disease, the concept of gene therapy broadened to have application in diseases other than those with a purely genetic cause. In this case, the delivered gene would encode a protein that is not missing or dysfunctional in the patient but that provides some therapeutic benefit. Some examples might be a tumour suppresser gene to treat cancer or a gene for a cytotoxic protein to kill cancer cells, the tyrosine hydroxylase gene to treat Parkinson's disease, or a gene encoding an immunostimulatory protein for treatment of cancer or infection.

Thus the concept is a simple one. Putting this into practice, however, is far from simple and this will become apparent in the following discussion. Although identification and cloning of

potentially therapeutically useful genes has been highly successful, delivery of the genes to the cell remains a major limiting factor. Delivery vectors are broadly divided into viral and non-viral; the discussion here will be mainly concerned with non-viral vectors.

2.2 A Brief History

The concept of gene therapy is widely thought to have come about relatively recently. In fact, the idea that one could manipulate genes to treat human disease has been around for some decades now. In 1944, Avery *et al* found that a non-virulent form of *Pneumococcus* could be transformed into an infective form using extracted nucleic acids from a virulent form [Avery *et al*, 1944]. This was essentially the first suggestion of a link between DNA and genetic information, and this revelation is widely cited as the foundation-stone of molecular genetics and, as such, of the concept of gene therapy. Over the decade following this discovery, nucleic acids moved from being considered as largely uninteresting molecules to being the most likely candidate for the gene [Lederberg, 1994] culminating in the elucidation of the structure of DNA [Watson and Crick, 1953]. Even so, the possibility of molecules other than DNA playing a significant role in genetics had not been entirely dismissed [Lederberg, 1994] and it could be argued that rigorous proof came only in the 1960's when DNA synthesised *in vitro* was shown to be biologically active [Lederberg, 1994]. Throughout the 1950's and 60's the ability of viruses to stably transform inheritable changes was observed, first by bacteriophages, and later by animal viruses including Rous Sarcoma virus, and the papovaviruses SV40 and polyoma [Wolff and Lederberg, 1994]. By the mid to late 1960's, forward-looking researchers were beginning to see the use of these viruses as a mechanism to deliver therapeutically useful genes as a possibility [Friedmann, 1992], in particular, Tatum provided an explicit account of the concept of viral gene therapy in 1966 [Tatum, 1966]. At this stage, though the concept of gene therapy was inviting, not only in the treatment of monogenic disease, but also in cancer [Tatum, 1966], the existent technology precluded the possibility of integrating therapeutically useful genes into a virus vector. The 1970s saw a technological revolution in molecular biology with the discovery of restriction enzymes at last providing the tools needed for genetic engineering, and allowing gene therapy to become a serious possibility. In 1973, a new calcium phosphate transfection procedure was developed which allowed DNA to be introduced efficiently and functionally into human cells *in vitro* [Graham and Van der Eb, 1973]. In 1980, Cline reported the successful introduction of the human globin gene into the bone marrow of mice using an *ex vivo* technique involving calcium phosphate transfection [Cline *et al*, 1980]. At the time this experiment received much media attention and was described as "revolutionary" [Marx, 1980]. Cline followed up the study with the first human gene therapy trial. Bone marrow cells from thalassaemia patients were transfected *in vitro* with the human globin gene, and reintroduced into the patients [Friedmann, 1992]. The trial failed to induce any change in the patients. It was severely criticised on scientific, administrative and ethical grounds [Friedmann, 1992] and

sparked much debate on the ethics of gene therapy [Kolata and Wade, 1980]. During the early 1980s techniques were developed that enabled the genetic manipulation of retroviruses to incorporate therapeutically useful DNA. The high efficiency of this method of gene transfer to cells in culture generated increased interest and enthusiasm towards the possibility of human gene therapy, and towards the mid 1980s human gene therapy became accepted as a genuine possibility awaiting practical application. From 1989 onwards, increasing numbers of clinical trials of gene therapy and marker gene experiments were approved. In the late 1980s and early 1990s, a variety of new methods of transfection were explored including electroporation, new viral vectors and lipid based excipients. There has been a remarkable growth in research effort directed towards gene therapy since the early 1990s. This is illustrated by the growth in the number of research papers published in this field. Figure **21** shows the number of articles published, that appear on the Science Citation Index database, containing the phrase "gene therapy" in their title from 1981 to 1998.

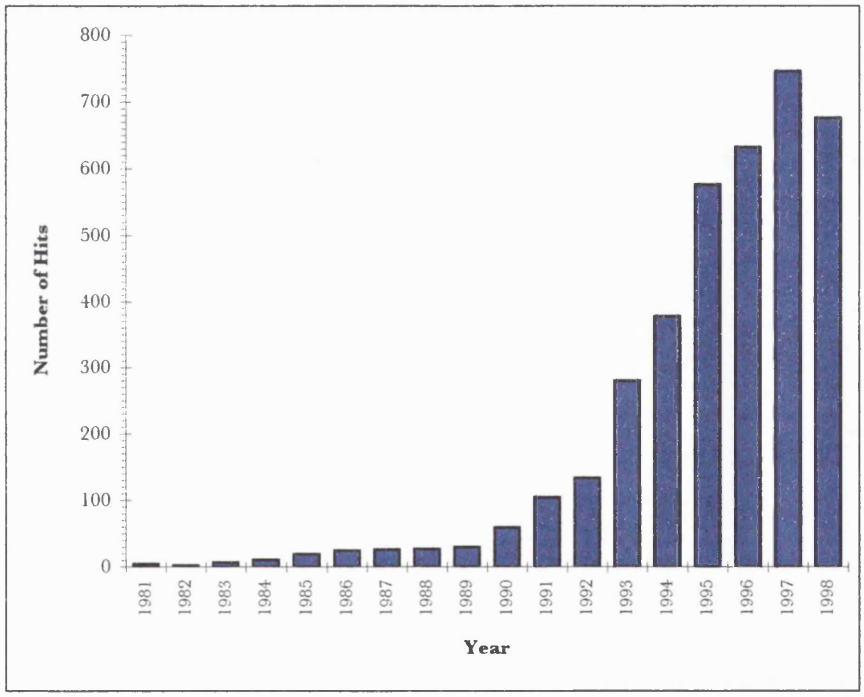


Figure 21 Articles containing "gene therapy" in their title 1981-1998, taken from the Science Citation Index database.

2.3 Germ-line Gene Therapy

Although all current gene therapy protocols are being performed on somatic cells, gene transfer could also be performed on germ cells. If the target cells are spermatozoa, ova or their precursors, the genetic modification will be passed on to the individual's descendants. This is referred to as germ-line gene therapy. If the target cell is somatic the modification will not be hereditary. There is, of course, an extremely tiny risk of inadvertent germ-line gene transfer

occurring *via* somatic gene therapy. Germ-line gene therapy is a controversial topic, largely due to the fact that it is not reversible. Ethical issues also surround its potential for misuse, such as for cosmetic purposes.

2.4 *In Utero* Gene Therapy

Advances in *in utero* diagnosis and gene delivery have led to the concept of prenatal gene therapy, an opportunity to treat disease *before* birth [Senut and Gage, 1999]. *In utero* gene therapy is also a controversial procedure, owing to many safety concerns and social and ethical issues [Billings, 1999; Senut and Gage, 1999]. Probably of greatest concern, in terms of safety, is the risk of inadvertent gene transfer to tissues other than the target tissue, particularly germ cells. The procedure does have potential advantages [Schneider and Coutelle, 1999; Senut and Gage, 1999]. Post-natal gene therapy may be too late to avoid irreversible disease manifestation, whereas gene delivery *in utero* may prevent the onset of disease. A developing foetus may be more amenable to uptake and permanent integration of foreign genes and organ stem-cell populations, inaccessible later in life, may be targetable. The immaturity of the foetal immune system may be more tolerant towards both vector and transgene and the size of the foetus means that fewer cells are targeted, thus less vector is required. The immaturity of the foetal blood-brain barrier may facilitate delivery to the brain.

2.5 Therapy with Antisense Oligonucleotides

Antisense oligonucleotides are sequences of around 15 to 20 nucleotides, complementary to specific sequences of DNA or RNA. They have been proposed as therapeutic entities because they are able to inhibit gene expression; thus there is the possibility to down-regulate the production of deleterious proteins. There is controversy over the mechanism of action of antisense oligonucleotides; a number of mechanisms have been put forward [Crooke, 1998; Israel and Domb, 1998]. These include activation of RNase H (causing arrest of transcription by breakdown of RNA) by formation of a heteroduplex double-stranded sequence with target mRNA, inhibition of translation by binding to the initiation region, and formation of a triplex with a specific DNA sequence which can cause inhibition of transcription. Antisense oligonucleotide therapy suffers the same major problem as gene therapy using DNA, the lack of efficient vectors for intracytoplasmic delivery. Antisense oligonucleotides can be modified such that they are more resistant to nucleases, for example by producing phosphorothioate analogues [Crooke, 1998].

2.6 Ethical Issues

It seems almost certain that at some stage in the future gene therapy will become a widely practiced technique in medicine. However, when gene therapy eventually does emerge as a fully developed branch of medicine, it seems unlikely that it will avoid media and thus public scrutiny

in terms of ethics and safety. It can be seen that germ-line and *in utero* gene therapy raise obvious ethical questions but consideration of ethical issues is important in gene therapy as a whole, more so than other emerging medical advances. Any new technology involving genetic manipulation tends to be greeted with apprehension and public perceptions of the relative risks and benefits of gene therapy will be important considerations in measuring its acceptability and consequent success.

3 What Diseases May be Treatable and With Which Genes?

3.1 Overview

In theory, one can envisage treating *any* disease with gene therapy. It is usually not too difficult to imagine a protein that may confer some benefit to a patient if it were expressed within a specific tissue or even systemically. In practice certain diseases are more suitable candidates for gene therapy, the obvious examples being genetic disorders where gene therapy is often the only treatment that may promise a complete cure. Diseases not treatable or curable by conventional therapies such as many cancers and AIDS, are other examples. Recent clinical trials statistics [http://www.wiley.com/wileychi/genmed/clinical/diseases.html; 1st June 1999] show that there are 380 gene therapy clinical trials protocols, involving 3173 patients, approved worldwide; this is illustrated in figure **22**.

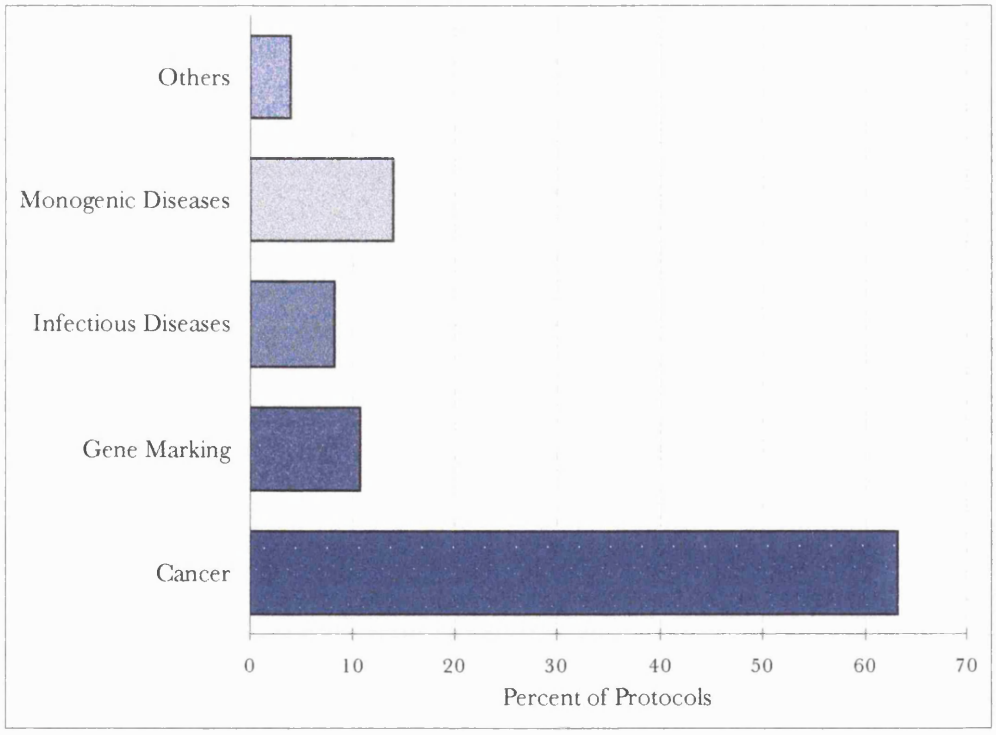


Figure 22 Gene therapy protocols per type of indication.

3.2 Cancer

It can be seen from figure **22** that the majority of current gene therapy trials are aimed at treating cancer. Cancer represents an attractive target for gene therapy. The lack of recent significant advances in conventional therapies, that is surgery, radiotherapy and chemotherapy, has obviously led to a greater interest in alternative therapeutic approaches. The possibility of direct injection of the gene medicine into a tumour may also have influenced research effort towards treating cancer, since this negates the need for selective targeting *via* the systemic circulation, a problem yet to be overcome satisfactorily. Another consideration that potentially makes cancer a less demanding target for gene therapy is that once the cancer has gone the genetic modification may no longer be required. This is in contrast to the situation with monogenic diseases where the formulation may have to be administered for the life of the patient if prolonged expression of the gene cannot be achieved.

Strategies for the gene therapy of cancer

There are numerous strategies for the gene therapy of cancer [Dachs *et al*, 1997; Hersh and Stopeck, 1998; Culver *et al*, 1995; Blaese, 1997]. Perhaps the most obvious approach is the introduction of a gene encoding a protein toxic to tumour cells, so-called suicide genes. Another approach is *gene-directed enzyme prodrug therapy* (GDEPT). Here, a gene that encodes a prodrug-activating enzyme is introduced, for example herpes simplex virus thymidine kinase (HSV-TK). This enzyme phosphorylates non-toxic nucleoside analogues such as ganciclovir which then act as chain terminators, killing dividing cells [Dachs *et al*, 1997]. One could also introduce genes that render cancer cells more sensitive to conventional chemotherapy, or attempt to protect vulnerable non-cancerous tissues against the toxic effects of systemic chemotherapy. For example, insertion of multi-drug resistance genes into haematopoietic stem cells would allow bone marrow to tolerate higher chemotherapeutic doses [Culver *et al*, 1995]. This could be accomplished using an *ex vivo* approach *i.e.* bone marrow transplantation with transduced stem cells.

Many approaches are aimed at stimulating the immune system against the tumour. This may involve genes expressing cytokines such as IL-12 [Mendiratta *et al*, 1999], or genes for tumour antigens, so-called DNA vaccines [Hersh and Stopeck, 1998]. Another approach is mutation correction or compensation. This may involve introduction of a tumour suppresser gene such as *p53*, activation of repair processes or blockage of oncogene activity.

Targeting

Some approaches require the gene to be expressed exclusively in tumour tissue, therefore strategies for selective delivery or selective expression are required [Dachs *et al*, 1997]. Tumour selective delivery *via* the systemic circulation may be possible by constructing a vector that

includes a targeting ligand capable of binding predominantly to tumour cells, for example, by taking advantage of the over-expression of certain receptors such as epidermal growth factor receptors and folate receptors seen in many tumours. Targeted expression may be possible if tissue specific promoters are included within a gene construct to allow expression of the transfer gene only in the tissue of interest. The simplest method of selective tumour targeting is direct injection of the formulation into the tumour mass. However, this may be impractical if there is more than one tumour mass or if the tumour(s) are not easily accessible.

The bystander effect

It has been observed within *in vivo* tumour models that complete ablation of the tumour can occur without 100% of the cells receiving the transgene. This is termed the bystander effect and is thought to occur by the passage of the therapeutic protein through gap junctions [Blaese, 1997]. This is an important observation since it is not possible to achieve anything near 100% gene transfer *in vivo* with current methods of delivery. A recent study using a GDEPT approach (using *HSV-TK* and ganciclovir) for retinoblastoma [Hayashi *et al*, 1999] reported 90% cell killing when only 50% of cells expressed *HSV-TK* in an *in vitro* model.

3.3 Monogenic Diseases

The paradigm here is cystic fibrosis. Cystic fibrosis is caused by a defect in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene and consequent defective production of the CFTR protein, a cAMP-regulated Cl^- channel. The end result of this is impaired Cl^- transport at the apical surface of epithelial cells. This leads to abnormal secretions in the gut, liver, pancreas and reproductive tract, although the clinical picture is dominated by a chronic lung disease caused by abnormal mucus secretion and impaired mucociliary clearance leading to chronic bacterial infection. Gene therapy, at present, is aimed at delivering a normal copy of the *CFTR* gene to the lungs. This represents a different challenge from the gene therapy of cancer. One could imagine that the delivery of a gene to the epithelial cells of the lungs was perhaps one of the easier tasks set the gene therapy scientist given its accessibility. However, the lungs present a number of barriers to successful gene delivery. These are applicable to lung delivery for any condition and are described in general later (see discussion on pulmonary delivery). In particular, the thick secretions produced by CF patients provide a major physical barrier to access of the epithelial cell surface [Davies *et al*, 1998].

The first approved gene therapy clinical trial actually aimed at curing a disease was for adenosine deaminase (ADA) deficiency [Blaese *et al*, 1995], a disease caused by a defect in the ADA gene which leads to severe combined immunodeficiency (SCID). In ADA deficiency patients, deoxyadenosine, usually catabolised by adenosine deaminase, is converted to the toxic compound deoxyadenosine triphosphate. T cells have a high turnover of deoxyadenosine and

are particularly prone to its toxic effects and those of its metabolites, thus the immune system is disabled [Blaese *et al*, 1995]. This first trial provides an illustration of the approach to gene therapy of ADA deficiency; T cells were separated from the patients blood, proliferated in culture, transduced with the ADA gene and reinfused into the patients.

Other monogenic disease candidates for gene therapy include Duchenne-Boulogne muscular dystrophy, Gaucher's disease (glucocerebrosidase deficiency), mucopolysaccharidosis (β-glucuronidase deficiency), hyperammonemia (ornithine transcarbamylase deficiency) and familial hypercholesterolemia.

3.4 Infectious Diseases

Although gene therapy could be used to target a number of infectious diseases, to date, AIDS is the only one for which there have been clinical trials. In fact, this disease represents the next largest group of studies after cancer. The goal of gene therapy here is to stop HIV from replicating inside infected cells or prevent the virus from spreading to healthy cells [Blaese, 1997]. The delivered gene may, for example, encode an inactive version of a protein that HIV uses in replication, the altered protein would then disrupt viral replication.

3.5 Other Diseases

Gene therapy has the potential to be used in many other disease states including Parkinson's disease, Alzheimer's disease, diabetes, rheumatoid arthritis, asthma and cardiovascular disease. In Parkinson's disease, use of the tyrosine hydroxylase gene has been suggested [During and Ashenden, 1998]; tyrosine hydroxylase is the rate-limiting enzyme in dopamine biosynthesis. However, increased tyrosine hydroxylase activity would not prevent the ongoing disease process, so an alternative approach might be to use genes for neurotrophic factors with the aim of regenerating existing neurons [During and Ashenden, 1998]. Symptoms of Parkinson's disease occur when 80% of striatal dopaminergic neurons have been lost, hence minimal increase in dopamine production could be sufficient to ameliorate symptoms. The brain is difficult to target; it is not accessible (due to the skull and blood brain barrier), neurons are largely terminally differentiated post-mitotic cells and there is a lack of well-characterised, cell specific promoters. There would usually be a need for global gene delivery in Alzheimer's and most neurogenetic disorders [During and Ashenden, 1998]. Diabetes may be targeted by genes whose products stimulate pancreatic β-cell neogenesis, as although β-cells are largely eliminated, more so in type I, precursor cells still remain [Levine and Leibowitz, 1998]. Early intervention to locally ablate the autoimmune response in type I disease by expression of immunomodulatory molecules by islet cells is another approach [Levine and Leibowitz, 1998]. Gene therapy for cardiovascular disease has focused on the vascular response to injury following surgery such as bypass grafting and angioplasty which can limit the long term efficacy of these

techniques [Svensson and Schwartz, 1998]. Gene therapy for rheumatoid arthritis may involve the delivery of genes whose products are anti-inflammatory, immunomodulatory or both. A current trial is using the interleukin-1 receptor antagonist gene *IL-1ra* with some success [Reinecke *et al*, 1999].

3.6 Gene Marking

Note that about 10% of trials (figure **22**) involve so called *gene marking*. This term is used when the gene being introduced is not expected to confer any beneficial therapeutic effect and the study is being conducted only to analyse the fate of the gene or its product.

4 Routes of Administration

4.1 Overview

With the exception of the oral route, virtually every conceivable route of drug delivery has been used in a trial of some sort. Figure **23** shows the most common routes of administration being used in current trials [http://www.wiley.com/wileychi/genmed/clinical/routes.html; 1st June 1999]. The chosen route of administration will obviously depend on the target tissue and upon the method of delivery, although this may be limited by the technologies available.

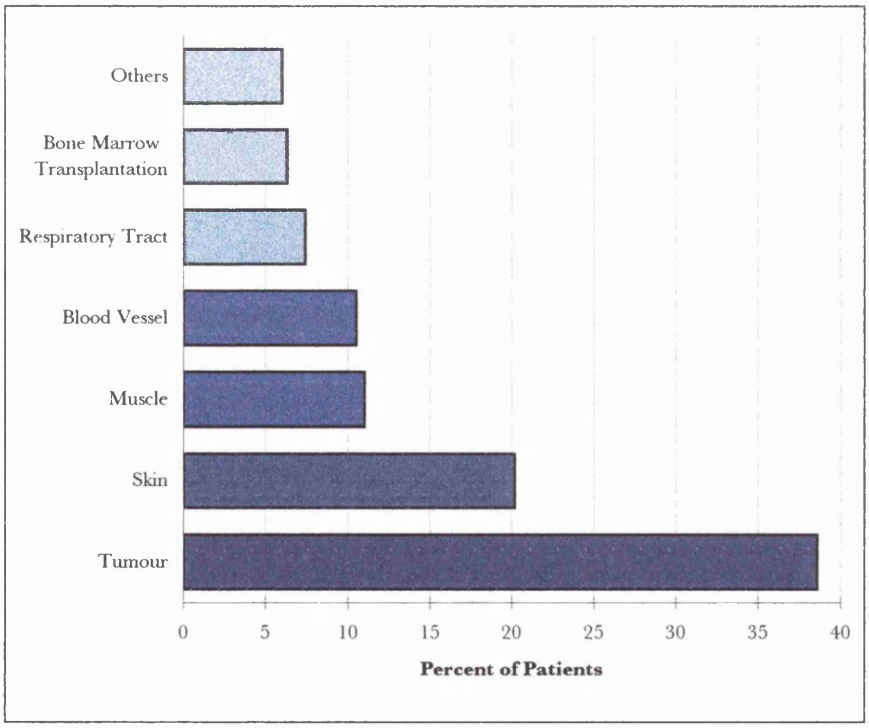


Figure 23 The routes of administration being used in current gene therapy trials expressed in terms of per cent of patients receiving the formulation by that route. Approximately half the current trials are using *ex vivo* techniques; in these cases the route of administration refers to the route by which the transgenic cells are administered.

4.2 Systemic Delivery

Probably the ultimate goal of gene therapy research is the production of a gene medicine that is stable in the circulation and can therefore be intravenously injected, possesses some sort of targeting system allowing tissue specific expression, and is able to release the DNA efficiently at the desired location. At present, the first step, a long-circulating gene delivery system, is not available. Naked DNA injected intravenously is rapidly eliminated from the plasma due to extensive uptake by the liver and rapid degradation by serum and tissue nucleases [Takakura and Hashida,1998]. Hepatic uptake is highly efficient, clearance being nearly identical to plasma flow through the organ. The uptake is thought to be by non-parenchymal cells *via* scavenger receptors for polyanions. No significant gene expression is observed [Takakura and Hashida, 1998]. Liposome-DNA complexes are also rapidly cleared by the liver. Hydrophobic particles are typically coated by specific plasma proteins (opsonisation) and taken up by macrophages in the liver and spleen *i.e.* activation of the complement system. The complement-activating properties of a range of polymers used for gene delivery and cationic lipids and their complexes with DNA has been examined [Plank *et al*, 1996]. Strong activation is seen with poly-L-lysine (PLL), polyethylenimine (PEI), a polyamidoamine (PAMAM) dendrimer and dioctadecylamidoglycylspermine (DOGS), other cationic liposomes are weaker activators. Short-chain oligolysines are comparable to cationic lipids. The degree of activation by complexes is lower, and for PLL, PEI, PAMAM dendrimer and DOGS, is strongly dependent upon charge ratio. Surface modification of preformed PLL complexes with PEG considerably reduces complement activation. Complexes of DNA with cationic polymers also show short plasma circulation times [Pouton and Seymour, 1999]. Again, there is hepatic uptake but also evidence of accumulation in the skin and intestine, probably due to physical trapping of the complexes within the fine capillaries of these organs, possibly after binding to plasma proteins. Glycosylated cationic polymers have been widely studied as possible liver-selective carriers for DNA *via* the systemic circulation. Liver parenchymal cells possess asialoglycoprotein receptors, which have limited distribution in other tissues and which recognise galactose. A complex of radiolabelled DNA and galactosylated PLL rapidly accumulated in the liver of mice (80% of the injected dose within 5 minutes of injection) [Hashida *et al*, 1998]; of this, 70% was found in parenchymal cells. Uptake was selectively inhibited by pre-injection of excess Gal-bovine serum albumin, an asialoglycoprotein receptor ligand, indicating that the complex is being taken up by receptor-mediated endocytosis. Clearly systemic gene delivery is not an easy task; we have yet to jump the first hurdle, to develop a delivery system that is not cleared rapidly from the circulation.

4.3 Intratumoural Injection

It can be seen from figure **23** that almost 40% of patients enrolled in gene therapy trials are being given the formulation *via* the intratumoural route. This is largely a reflection of the fact

that many trials are being conducted in the area of oncology, but may also be biased by the fact that direct injection of the formulation into the target tissue is considerably easier than targeting cancer tissue *via* the systemic circulation. In this context, naked DNA has been shown to be more active than lipidic formulations [Yang and Huang, 1996]. This is possibly due to the high hydrostatic pressure within solid tumours, caused by poor lymphatic drainage and poor vascularisation, preventing adequate dispersion of the formulation [Pouton and Seymour, 1998] which may limit release of DNA from the complex.

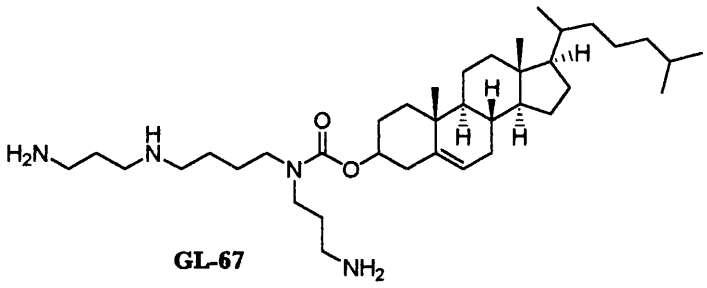
4.4 Intramuscular Injection

Gene transfer into muscle cells *in vivo* follows injection of naked DNA. This was first observed in the mouse in 1990 [Wolff *et al*, 1990]; reporter genes were injected directly into the mouse quadricep muscle and protein expression was detectable. Formulations of reporter genes with the hydrophilic polymers PVP and PVA show enhanced expression over naked DNA [Mumper *et al*, 1996], this may be due to enhanced dispersion and/or osmotic effects. A more detailed discussion concerning PVP-DNA formulations for muscle delivery will be presented later in this chapter. After direct injection of lipoplexes, gene expression is often localised to regions close to the injection site; this suggests that dispersion of the formulation within the muscle tissue is critical [Pouton and Seymour, 1998].

The finding that primary myoblasts (easily identifiable and isolable muscle stem cells) can be grown in culture and be stably transduced with recombinant genes further stimulated interest in muscle delivery. The modified myoblasts fuse with endogenous myofibres to become stably incorporated into muscle tissue [Svensson *et al*, 1996]. The modified cells can produce and secrete large amounts of recombinant protein and the rich vascular supply to skeletal muscle promotes the efficient access of secreted proteins into the systemic circulation. This approach, although successful in animal studies, was difficult to translate to human gene therapy [Svensson *et al*, 1996]. Human muscle appears to be significantly less receptive to translated myoblasts. The technique is also labour intensive and expensive. Despite this promising technique not yet fulfilling its potential, muscle remains an attractive tissue for gene delivery, not only for treatment of muscular disorders but for the treatment of other diseases where the muscle could act as a factory for therapeutic proteins [Mumper and Rolland, 1998]. This approach may be particularly useful in the treatment of serum protein deficiencies, and muscle may provide a useful site for the delivery of DNA vaccines where DNA encoding a foreign protein is used to stimulate an immune response. Here, only transient production of the protein may be required and the technique may be successful even with low efficiency, localised gene expression.

4.5 Pulmonary Delivery

Gene delivery to the lung is of interest for a number of reasons. The tissue is potentially more accessible than many other tissues; this may overcome problems with targeting. Further, cystic fibrosis is an attractive target for gene therapy; lung disease is the dominant manifestation of this condition and delivery of the *CFTR* gene to the lung may prove to be the best way to manage the condition. The lung may also be useful for the systemic delivery of genes. Furthermore, much is known about the fate of inhaled formulations in the lungs, largely through the development of asthma medicines. Delivery to the lung is, however, presented with significant barriers. Epithelial monolayers are polarised cells with characteristic tight junctions; the apical surfaces are tough, strengthened by actin filaments close to the plasma membrane and do not have extensive endocytic capacity [Pouton and Seymour, 1998]. The mucociliary clearance system of the respiratory tract is highly effective at excluding foreign material and, as such, is a major barrier to access of the lung epithelium by gene medicines. Mucus, secreted by goblet cells within the epithelium, provides a mechanical and diffusional barrier. In bronchiolar regions, cilia move the mucus and trapped particles up and out of the lung and alveolar macrophages clear particles deeper in the lung. Much work has been focused upon optimizing lipoplex formation for pulmonary delivery. In one study, a large series of lipo-polyamines were synthesized and their transfection efficiency was examined *in vivo* in mouse lung [Lee *et al*, 1996]. One cationic lipid in particular, **GL-67**, gave a 1000-fold increase in reporter gene expression over a naked DNA formulation.



GL-67

A formulation of **GL-67** and the *CFTR* gene, administered *via* the nasal epithelium, was evaluated in CF patients. However, correction of the electrolyte transport defects was not appreciably greater than from a formulation of naked DNA [Zabner *et al*, 1997]. **GL-67** was re-evaluated in transgenic CF mice [Jiang *et al*, 1998]. The study found that although **GL-67** mediated transduction of *CFTR* to airway epithelia, the process was relatively inefficient giving marginal correction of ion transport.

5 Methods of Delivery

5.1 Naked DNA

The direct injection of DNA not formulated with any delivery vectors may have application in certain situations. The low level expression seen after the direct injection of naked DNA into muscle tissue may be enough to achieve an immunological response and hence have application in the development of DNA vaccines. Expression may also be seen after direct injection of naked DNA into solid tumours.

5.2 Viral Vectors

Viruses are the professionals of gene delivery; they have evolved specific mechanisms to enter cells and express their genes and do so highly efficiently. Technology to substitute genes involved in viral replication for potentially therapeutic genes is well established, thus they are attractive as vectors for gene therapy. Over 70% of current trials are using viral gene delivery [http://www.wiley.com/wileychi/genmed/clinical/vectors.html; 1st June 1999] and the majority of these involve the use of retrovirus. Although at present viral vectors are generally more efficient than non-viral systems, there are a number of problems associated with the use of viral vectors, including immunogenicity, transient duration of gene expression, small capacity for foreign genes and the generation of sufficiently high titres (the number of infectious viral particles per ml) [Robbins *et al*, 1998; French Anderson, 1998; Friedmann, 1997]. Four types of virus have found use in clinical trials - retroviruses, adenoviruses, herpes-simplex viruses (HSV) and adeno-associated viruses (AAV) [Robbins *et al*, 1998]. Viruses must bind to extracellular receptors before they are internalised and most cell types possess receptors to which viruses can bind. The engineering of viral envelope proteins to include new components or to replace existing ones may allow targeting to different cells from those that the virus would naturally infect [Friedmann, 1997]. The different properties of different types of virus dictate how they may be used for gene delivery. Retroviruses can splice copies of the genes they carry permanently into the chromosomes of the cells they invade [Friedmann, 1997] which may allow long term expression of the gene. However, they usually require the cells to be dividing to stably infect them, thus an *ex vivo* approach is primarily used. In addition, they do not have the capacity for large genes such as the dystrophin gene [Robbins *et al*, 1998]. Adenoviruses can accommodate large DNA inserts, up to 35Kb, they are able to transduce a wide variety of cell types including non-dividing cells and can be grown to high titres [Robbins *et al*, 1998; Friedmann, 1997] however, they may only produce transient expression of the gene. Adenoviruses have been the vector of choice for cystic fibrosis and a variety of trials in oncology [French Anderson, 1998]. AAV is a human virus for which the majority of the population is seropositive; it is not associated with pathologies. The degree of infection of muscle, brain and

liver cells with recombinant AAV is reported to be high *in vivo* [Robbins *et al*, 1998]. AAV has a small capacity for foreign genes (about 4.8Kb of DNA) and production of viral particles is very labour intensive due to the lack of efficient packaging cells [French Anderson, 1998]. HSV is particularly able to transfect nerve cells and may have potential as a vector for delivery to the nervous system [Ho and Sapolsky, 1997]; is has a large capacity for DNA (up to 50Kb). Thus viral vectors have much potential as instruments for clinical gene delivery, however, they have disadvantages which may be difficult to overcome. In the future we may see more vectors incorporating components of viral and non-viral delivery systems, so-called "artificial viruses" that mimic the highly efficient processes of viral infection [Lehn *et al*, 1998]; there is much that we can learn from the mechanisms by which viruses overcome the biological barriers to gene transfer.

5.3 Non-viral Methods

Synthetic vectors

Synthetic gene delivery systems have developed as two broad classes of compounds, the cationic lipids and the cationic polymers. DNA complexes with cationic lipids have been named *lipoplexes* and the process of DNA delivery mediated by cationic lipids termed *lipofection* [Felgner *et al*, 1997]. Similarly DNA complexes with cationic polymers have been named *polyplexes* and cationic polymer mediated DNA delivery termed *polyfection* [Felgner *et al*, 1997]. Further, the term "interpolyelectrolyte complex (IPEC)", which in general refers to any complex of oppositely charged polyions, has been used to describe DNA-polycation complexes [Kabanov and Kabanov, 1995]. Synthetic vectors are most relevant to the work in this thesis and a detailed account of the most important synthetic vectors will be presented in the next section.

Physical methods

Microinjection

This is the direct injection of DNA into the nucleus of the target cell [Sih *et al*, 1994]. The technique is highly labour intensive and has little application *in vivo* as a large number of cells would need to be injected; this is not practicable.

Particle Bombardment

In this technique, DNA is coated onto the surface of 1-3 micron diameter gold or tungsten beads. The particles are accelerated by an electric discharge device or gas pulse and fired at the tissue [Schofield and Caskey, 1995]. The gene-coated particles penetrate the plasma membrane of cells and deliver the gene to the cytosol. The technique has been applied *in vitro* and *in vivo* with variable success depending on the tissue.

Electroporation

In this technique an electric field is applied to the cell membrane causing the formation of hydrophilic pores (*electropores*) in the lipid matrix. The electropores are relatively stable, lasting from seconds to minutes [Sukharev *et al*, 1994] and may be further stabilised by the involvement of membrane proteins. Upon pulse termination electropores eventually reseal, although small residual leaks may remain indefinitely, unless there is repair by membrane turnover. These electric pulses lead to cellular uptake of DNA. The mechanism of this phenomenon is poorly understood, the apparent size of the electropores is considerably smaller than a typical piece of DNA. Electroporation appears to be biologically non-specific and has been applied *in vitro* and *in vivo*. Its major limiting factor may be hindrance for the access of DNA to the membrane. Recently an electroporation method has been shown to be highly efficient in transfer of reporter and therapeutic genes to muscle in a variety of species *in vivo* [Mir *et al*,1999]. Electroporation is a promising technique; there are currently two protocols under way utilising electroporation as the gene transfer method [http://www.wiley.com/wileychi/genmed/clinical/vectors.html; 1st June 1999].

Calcium phosphate precipitation

This is one of the earliest methods for the delivery of DNA to cells *in vitro* [Graham and Van der Eb, 1973]. DNA is precipitated with calcium phosphate and the precipitate is applied to cell monolayers. The technique has a low transfection efficiency but proved to be a useful catalyst for the study of cellular DNA uptake and the development of gene delivery systems. The mechanism is thought to involve the uptake of particulate Ca^{2+}-DNA complexes by non-specific endocytosis. Efficiency of uptake is dependent upon the quality of the precipitation reaction and the size of the precipitate formed. Many cell types are resistant to this form of gene transfer [Ledley, 1995].

6 Synthetic Vectors

6.1 Lipidic Vectors

These represent the best characterised gene delivery systems, largely due to their success in mediating transfection *in vitro* and the fact that they are easily produced by short, high yielding syntheses. The most successful systems are cationic, consisting of a lipid with a cationic or polycationic head group. These lipids self-assemble in aqueous solution forming microscopic structures such as bilayers, micelles and liposomes. They fall into two broad categories:

1. Those with a monovalent head group which typically form liposomes often with the aid of a neutral co-lipid such as dioleoyl-L-α-phosphatidylethanolamine (DOPE, figure **24**), some examples are illustrated in figure **25.**

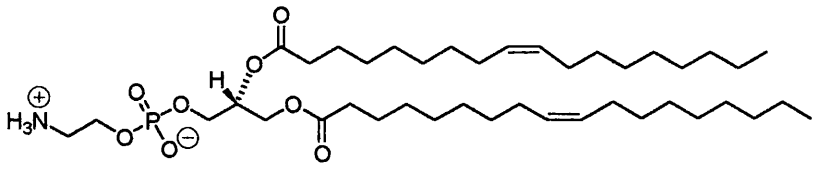


Figure 24 Dioleoyl-L-α-phosphatidylethanolamine

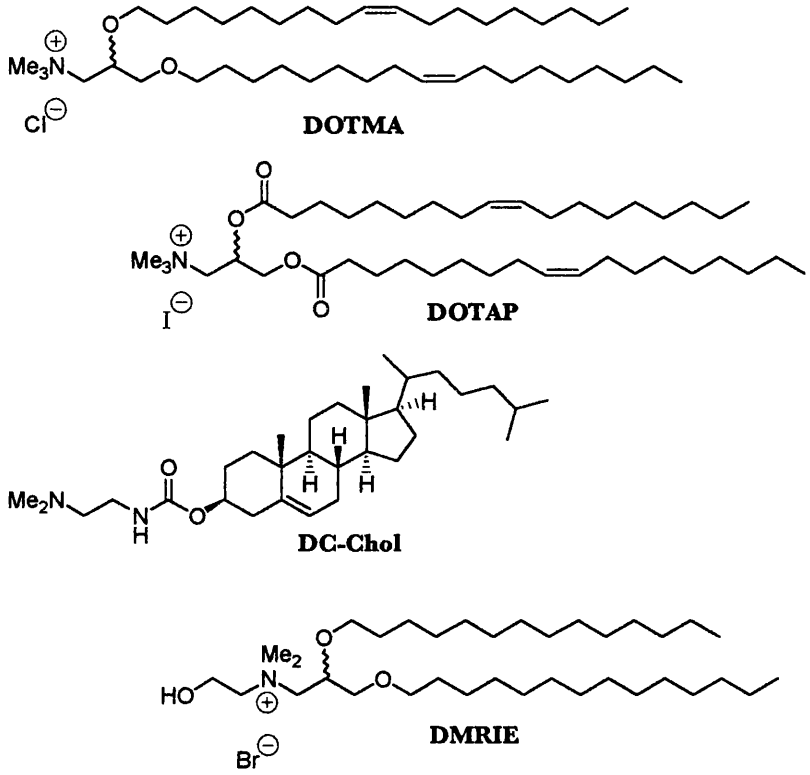


Figure 25 Some monovalent cationic lipids, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP), 3β-[N-(N',N'-dimethylaminoethyl)carbamoyl]cholesterol (DC-Chol) and N-(1,2-dimyristyloxypropyl)-N,N-dimethyl-N-(2-hydroxyethyl)ammonium bromide (DMRIE).

2. Those with a polycationic head group, the lipopolyamines, some examples of which are illustrated in figure **26.** Lipospermines are reported to form tubular micelles in aqueous solution [Remy *et al*, 1998].

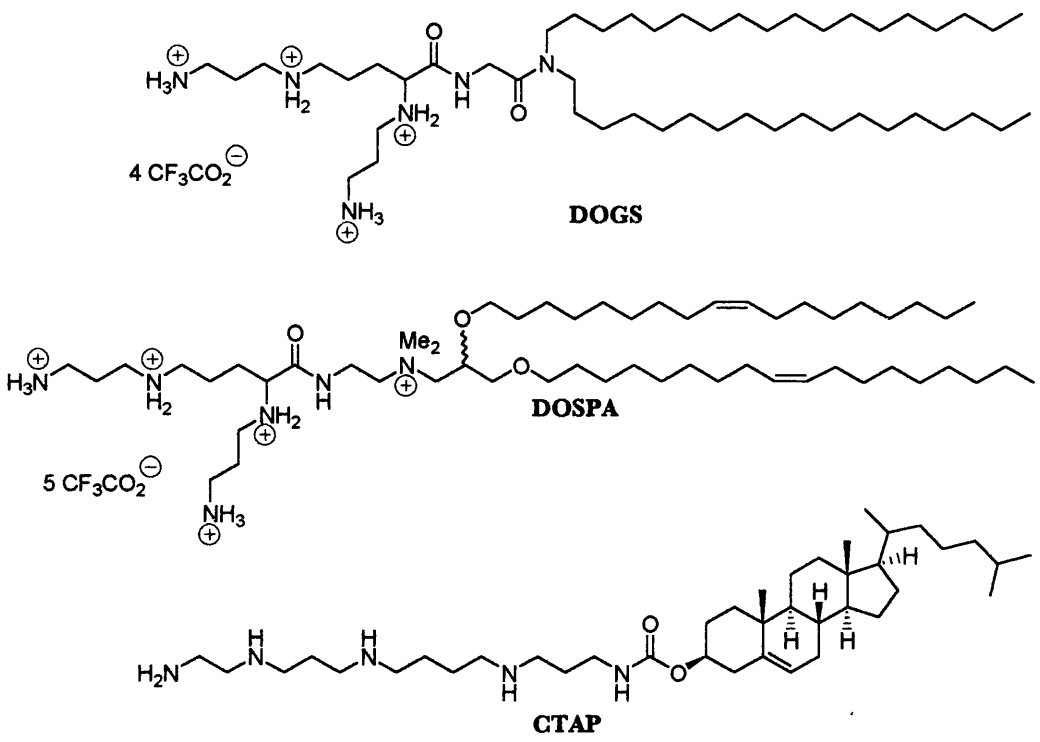
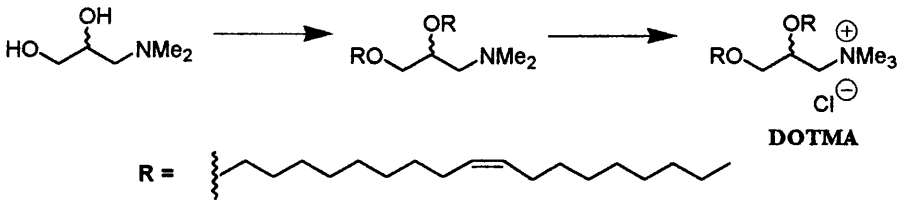


Figure 26 Lipids with a polycationic head group, dioctadecylamidoglycylspermine tetrakistrifluoroacetic acid salt (DOGS), 2,3-dioleyloxy-N-[2-(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and N^{15}-cholesteryloxycarbonyl-3,7,12-tri-azapentadecane-1,15-diamine (CTAP).

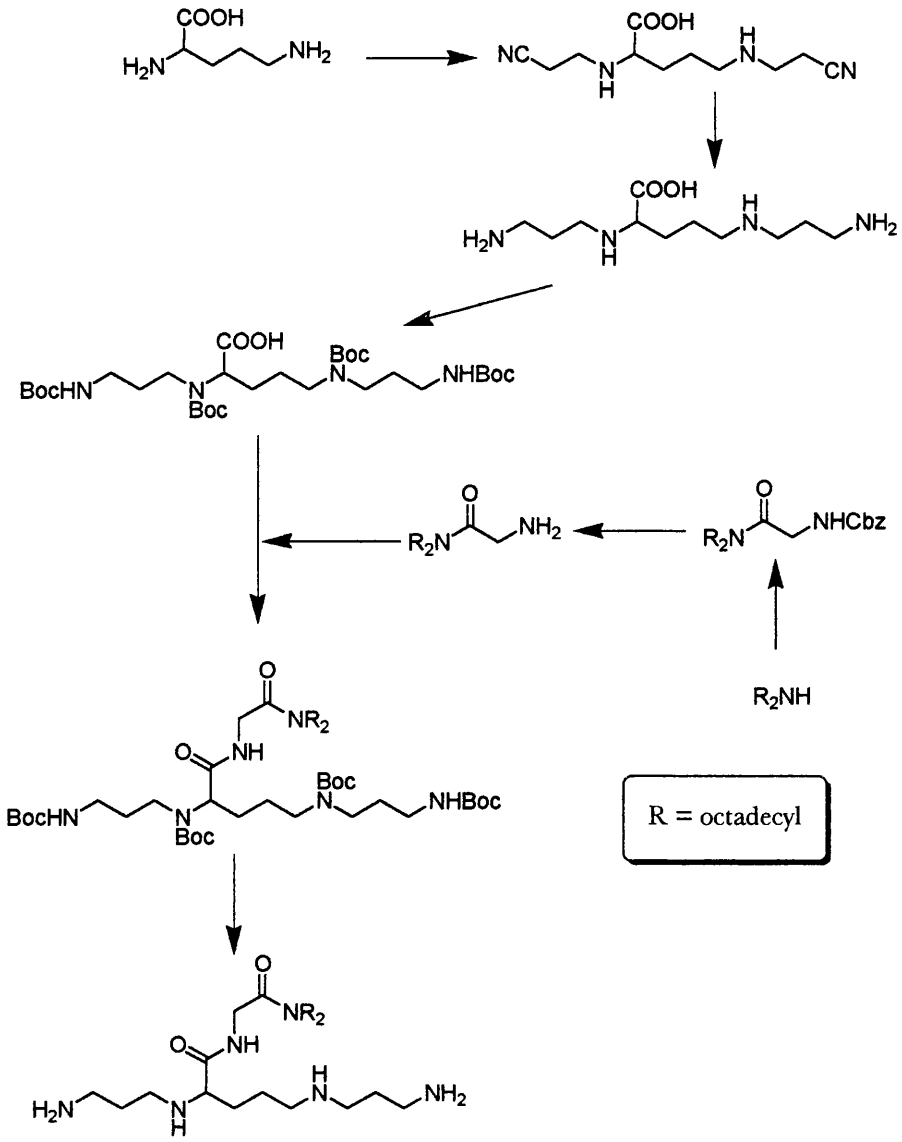
Synthesis of cationic lipids

DOTMA has been synthesised *via* a simple two step procedure [Felgner *et al*, 1987], illustrated in scheme **23**. It can be seen that the DOTMA analogues DOTAP and DMRIE can also be synthesised using similar procedures.



Scheme 23 Synthesis of DOTMA.

Synthesis of the polycationic lipids is a little more demanding. For example the synthesis of DOGS *via* a multistep convergent synthesis is illustrated in scheme **24** [Behr *et al*, 1989]. Note that the cyanoethylation then reduction of lysine provides a simple method for the synthesis of branched spermine derivatives.



Scheme 24 Synthesis of DOGS.

DC-Chol can be synthesised by simply coupling cholesterol chloroformate to N,N-dimethyl ethylenediamine [Gao and Huang, 1991]. Efficient syntheses of a number of polyamine analogues of DC-Chol, including CTAP, using cholesterol chloroformate have recently been reported [Cooper *et al*,1998].

Preparation of liposomes

Although some cationic lipids may self-assemble in aqueous solution to form microscopic structures, the formation of liposomes often requires some energy input into the system. Typically this involves vortexing, extrusion, sonication or homogenisation-microfluidisation of multilamellar vesicle suspensions prepared by the hydration of thin lipid films in aqueous solutions [Lasic and Templeton, 1996].

Transfection using lipidic systems

Since Felgner and co-workers [Felgner *et al*, 1987] showed that DNA complexes with lipidic vectors have high transfection efficiency *in vitro*, there have been hundreds of reports of the highly successful use of lipidic formulations for the transfer of DNA *in vitro* and there are many reviews covering this area [for example: Lasic and Templeton, 1996; Mahato *et al*, 1997; Remy *et al*, 1998; Miller, 1998]. The success of lipidic formulations has led to the widespread application of these systems as *in vitro* research tools and a number of systems are available commercially, some examples are given in table **4**.

Trade name and manufacturer	**Formulation**
Lipofectin, Gibco	DOTMA/DOPE 1:1
DOTAP, Boehringer Mannheim	DOTAP
Lipofectamine, Gibco	DOSPA/DOPE 3:1
Transfectam, Promega	DOGS

Table 4 Common commercial lipidic transfection agents.

Whilst these systems have been a great success for delivering DNA to cells in culture, their performance for the transfer of DNA *in vivo* has been somewhat disappointing and moreover, there is poor correlation between *in vitro* and *in vivo* activity. There are a number of possible reasons for this. Plasmid-lipid complexes often aggregate over time in the presence of salt or serum proteins [Mahato *et al*, 1997]. In particular, interaction of positively charged complexes with serum proteins and other extracellular matrix components may lead to aggregation or premature release of the DNA. As described earlier, the complexes are prone to opsonisation, activation of complement and subsequent reticuloendothelial clearance upon IV administration. There have, however, been a number of reports of successful gene transfer *in vivo* using cationic lipids *via* various administration sites [reviewed in Lasic and Templeton, 1996; Miller, 1998].

Transfection mechanism for lipidic systems

Liposome-DNA complexes are thought to enter cells *via* an endocytotic mechanism [Zabner *et al*, 1995] and there have also been suggestions of the complex entering the cell *via* plasma membrane fusion [Mahato *et al*, 1997]. Zabner *et al* have studied the mechanism and found that the overall process of cationic lipid-mediated gene transfer is inefficient despite most cells taking up the DNA. The study suggests that the limiting factors are release of the complex from the endosome and translocation of the DNA from the cytoplasm to the nucleus. The majority of delivered DNA accumulated in perinuclear endosomes. Release of the DNA from the complex is important, as free plasmid injected directly into the nucleus is expressed whereas plasmid-lipid complexes are not [Zabner *et al*, 1995]. The release of DNA from the complex has been studied

[Xu and Szoka, 1996]. It was found that cytoplasmic-facing components of the plasma membrane such as phosphatidylserine cause rapid release of DNA from the complex. The authors suggest that, once the complex is internalised by endocytosis, destabilisation of the endosomal membrane occurs. This induces the formation of a charge neutral ion pair between the cationic and anionic lipids of the endosomal membrane causing release of the DNA into the cytoplasm. Uptake of DNA into the nucleus is thought to involve an active transport process involving nuclear localisation signal proteins [Mahato *et al*, 1997].

6.2 Polymeric Vectors

A number of polymers, having various structures and properties, have been used in gene delivery experiments and the most important will be considered in turn. With the exception of PVP and PVA, which are neutral polymers, they are all polycationic.

Polyethylenimine

Polyethylenimine (PEI) is a highly branched polyamine having two methylenes between each nitrogen, synthesised by the acid-catalysed polymerisation of aziridine. The general structure is depicted in figure **27**.

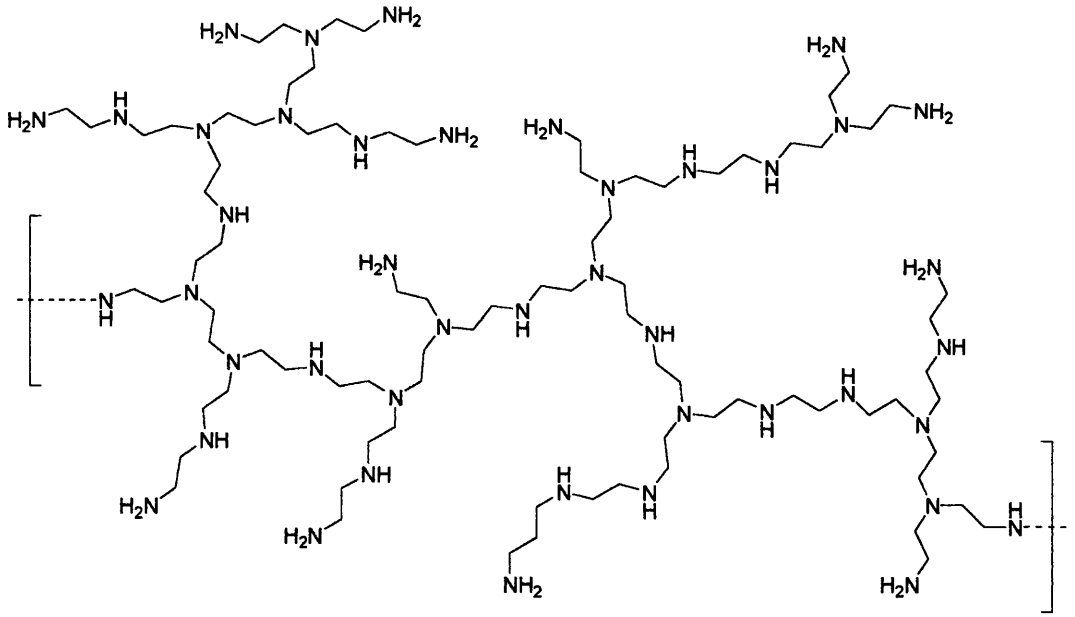


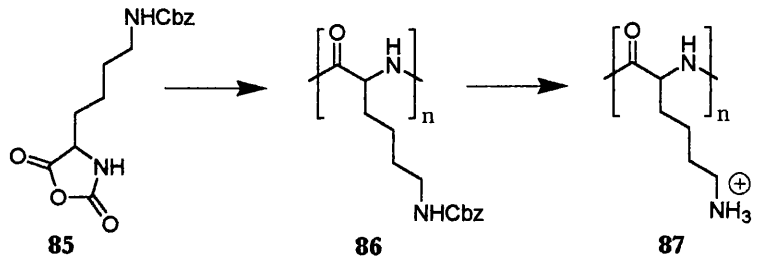
Figure 27 Polyethylenimine

PEI forms complexes with DNA *via* electrostatic interactions, it is around 20% protonated at physiological pH [Remy *et al*, 1998]. The transfection ability of PEI (800KDa and 50KDa) has been evaluated and compared with that of lipidic systems [Boussif *et al*, 1995]; it was found to be comparable to, or better than, lipopolyamines in mediating the transfer of a luciferase

reporter gene in a number of cell lines. Luciferase gene transfer *via* intracerebral injection into newborn mice was also reported to be successful. The authors attribute the efficiency of PEI-plasmid complexes on an extensive lysosome buffering effect, so-called "proton sponge" behaviour. At pH 5, protonation of PEI increases from around 20% to 45% [Remy *et al*, 1998], this property allows the polymer to accumulate protons in the endosome. The protons are pumped in by the endosomal ATPase in a process which is coupled to an influx of chloride ions. There is a net increase in the ionic concentration within the endosome and the polymer swells due to charge repulsion within its structure [Remy *et al*, 1998]. The authors postulate that this effect may cause endosomal disruption and inhibit the action of lysosomal nucleases. PEI has been shown to promote delivery of DNA to the nucleus [Pollard *et al*, 1998]. The study showed that PEI and, to a lesser extent PLL, enhanced transgene expression when the complexes were injected into the cytoplasm of a number of mammalian cell lines, no enhancement was seen with cationic lipids. The study also showed that cationic lipids prevented expression when the complex was injected into the nucleus, this was not the case for PEI or PLL complexes. How significant these finding will be in terms of future novel vector design remains to be seen but the study does highlight the fact that the fate of the complex at the sub-cellular level is an important consideration.

A linear 22 kDa form of PEI, ExGen 500, has been shown to be more efficient than cationic lipids in mediating transfection of reporter genes to CF epithelial cell lines and normal trachea cells *in vitro* [Ferrari *et al*, 1997]. ExGen 500 was also more efficient than DOGS in mediating transfection of reporter genes to the lungs of newborn rabbits [Ferrari *et al*, 1997]. The authors suggest the high transfection efficiency may be a result of the "proton sponge" behaviour of the polymer.

Poly-L-lysine



85 **86** **87**

Scheme 25 Synthesis of poly-L-lysine.

PLL **87** is synthesised by polymerisation of the N-carboxyanhydride (NCA) of Cbz-protected L-lysine **85** initiated with an amine nucleophile such as diethylamine to yield Cbz-protected PLL

86 (scheme **25**); the Cbz protection is removed from the polymer typically with HBr in glacial acetic acid. The primary amino groups are protonated at physiological pH and the polymer is thus able to interact electrostatically with DNA. PLL-DNA complexes have been extensively studied and are well characterised [Shapiro *et al*,1969; Wolfert and Seymour, 1996; Pouton *et al*, 1998]. PLL is able to mediate transfection *in vitro*, but typically requires an endosomolytic agent such as chloroquine [Pouton *et al*, 1998]. There are many problems associated with the potential use of PLL *in vivo*, not least, toxicity; it causes activation of complement and hepatic uptake. Further, aggregation of particles is a significant problem; PLL-DNA complexes are only soluble up to about 20μg/ml DNA [Wagner, 1998]. The size of DNA-PLL complex particles was found to be dependent upon the molecular weight of the PLL, PLL 224500 produced particles with diameters ranging from 120-300nm (as determined by atomic force microscopy) [Wolfert and Seymour, 1996], whereas PLL 3970 produced particles in the 20-30nm range. Complexes formed using lower molecular weight PLLs showed significantly decreased cytotoxicity *in vitro* [Wolfert and Seymour, 1996]. In attempts to improve the properties of PLL for *in vivo* delivery, PLL has been modified with many functionalities including targeting ligands and hydrophilic polymers [Wagner, 1998; Zauner *et al*, 1998]. The discussion below gives examples of the modification of PLL with hydrophilic polymers; a very large number of targeting ligands have been conjugated to PLL, these are mainly aimed at receptor-mediated delivery and include galactose to target the asialoglycoprotein receptor of hepatocytes, folic acid for folate receptors on tumour cells and transferrin for the ubiquitous transferrin receptor [Wagner, 1998; Zauner, 1998].

Hydrophilic copolymers

A number of block and graft copolymers of a polycation and a hydrophilic polymer have been prepared with a view to use in gene delivery. Hydrophilic polymers are used in an attempt to improve the biopharmaceutical properties of DNA-polycation complexes; the complexes with these copolymers have a greater water solubility and may have a reduced clearance and toxicity. Moreover, the use of hydrophilic constructs may allow the formulation of larger amounts of DNA than is possible with polymers such as PLL and PEI, whose complexes with DNA tend to aggregate at high DNA concentration. Many constructs have used PEG and given that the novel vectors described in this thesis are mPEG based, it will be useful here to summarise some properties of PEG.

Properties of Poly(Ethylene Glycol)

PEG has some interesting properties that have led to its use in a number of biomedical applications. PEG has extremely low toxicity [Pang, 1993] and is non-immunogenic. It has been approved by the FDA for internal consumption and can be safely administered intravenously. Small PEGs of molecular weight less than 400Da may exhibit some toxicity. It

has excellent solubility in aqueous solutions but also in many organic solvents including dichloromethane and toluene. It is not soluble in diethyl ether or in hexane, a property that can be useful in the isolation of PEG conjugates. Its presence in aqueous solutions has no deleterious effect on protein conformation or enzyme activity [Zalipsky, 1995]. A key property is the incompatibility of PEG with other polymers. PEG is effective at excluding other polymers from its presence in an aqueous environment; at high concentration a two phase system can be formed by solution of PEG and dextran for example [Harris, 1992]. Low toxicity and immunogenicity, and some properties of PEG-modified proteins are linked to this behaviour. These systems can also be useful in the purification of biological materials, as PEG can be used to precipitate proteins and nucleic acids. Further, partitioning of materials between two phase systems can be controlled by varying the phase components, for example in a PEG-dextran system, increasing PEG molecular weight acts to drive proteins into the dextran-rich phase.

PEG has been studied extensively in terms of modification of proteins and drugs by covalent attachment. Molecules to which PEG is attached usually remain pharmacologically active [Harris, 1992] but PEGylation affects certain properties of the molecule; these effects are often desirable ones. PEG solubilises other molecules, improves the water solubility of lipophilic organic compounds and the solubility of proteins in organic solvents. It renders proteins non-immunogenic, alters the pharmacokinetics of molecules and may aid the movement of molecules across cell membranes [Harris, 1992]. The most commonly used PEG molecular weights for biotechnical and biomedical applications are 2000-20000Da. The monomethyl ethers are commonly used to avoid cross-linking.

PEG-PLL block copolymers

An A-B type block copolymer of PEG and PLL (figure **28**) has been synthesised by polymerisation of the NCA of Cbz-protected L-lysine initiated with α-methoxy-ω-amino PEG 10000 [Wolfert *et al*, 1996]. The copolymer formed self-assembling complexes with DNA. The complexes showed less surface charge than corresponding PLL-DNA complexes reaching a zero value of zeta potential at a higher charge ratio. Atomic force microscopic analysis showed that the complexes have an extended form with relatively thick linear and toroidal strands in contrast to the discrete, roughly spherical particles of PLL-DNA complexes. The authors suggest the unusual conformation of the complexes is possibly due to interaction of the polycationic block with DNA giving a structure with the PEG orientated outwards; with the inability of PEG to form intramolecular hydrogen bonds, possibly requiring hydrogen bonding to water for stability, the whole structure may be prevented from collapsing by the hydration requirements of PEG. Cytotoxicity was comparable to that of PLL-DNA complexes. The PEG-

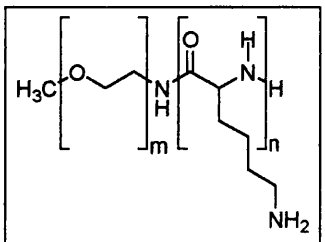


Figure 28 PEG-PLL block copolymer.

PLL complexes showed a significantly increased ability to transfect 293 cells (a human transformed foetal kidney cell line) with the β-Gal reporter gene compared to PLL. Optimal activity was at a charge ratio of around 1.5. It is suggested that this enhancement of gene transfer may be due to membrane activity or dehydrating fusogenic properties of PEG [Wolfert *et al*, 1996].

Another study using an A-B type block copolymer of mPEG 4300 and PLL 2500 [Katayose and Kataoka, 1997] examined the thermal melting curve of DNA complexed with PEG-PLL and with PLL alone, and the nuclease resistance of the complexes. The PEG-PLL copolymer produced a higher stabilisation of DNA structure compared to PLL, which the authors attribute to the compartmentalization of DNA into the microenvironment of PEG with low permittivity. Moreover, the copolymer-DNA complex, found to be *ca.* 50nm in diameter (by dynamic light scattering), showed high resistance to DNase I attack.

mPEG grafted poly-L-lysine

mPEG bearing a terminal carboxyl group was prepared from mPEG-NH_2 by reaction with succinic anhydride [Toncheva *et al*, 1998]. The carboxyl groups were coupled to amino groups of PLL 20000 using 1-ethyl-3-[(dimethylamino)propyl]carbodiimide hydrochloride (EDC) and the resulting copolymer purified by dialysis. Copolymers were prepared with 5 and 10 mole% mPEG 5000 and 12000. In contrast to the PEG-PLL block copolymer [Wolfert *et al*, 1996], these graft copolymers produced discrete and well-defined particles as judged by atomic force microscopy. Copolymers with the longer mPEG chains showed particularly effective shielding of surface charge of the complex as indicated by zeta potential measurements. The complexes were considerably more water soluble than PLL-DNA complexes. Transfection efficiency (293 cells) was greater for copolymers with longer mPEG chains or higher percentage mPEG grafting suggesting that possible membrane activity of PEG was promoting DNA entry into the cytoplasm [Toncheva *et al*, 1998]. In this study, PLL grafted with dextran (1000Da) and with polyHPMA (4400Da) were also prepared.

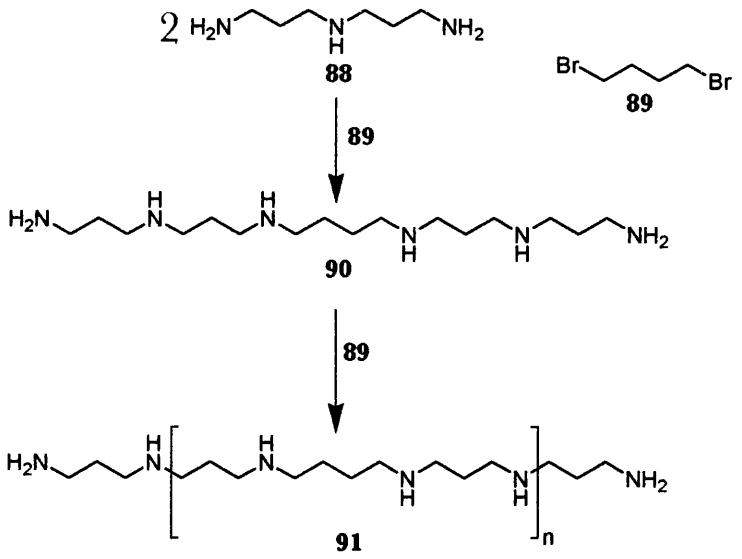
mPEG grafted PLL has been prepared by conjugating mPEG 500 carboxylic acid to the amino residues of PLL by generating the acid chloride with thionyl chloride and allowing this to react with PLL in DMSO [Choi *et al*, 1998]. Copolymers with 5, 10 and 25 mole% mPEG were prepared and purified by dialysis. The amount of mPEG grafting was determined from the ^1H NMR spectra by using the ratio of the mPEG CH_2 signal to the PLL CH_2 signals. The molecular weight of the PLL used was 25000Da. The mPEG grafted PLL showed a 5-30 fold increase in transfection efficiency in Hep G2 cells compared to unmodified PLL. Chloroquine increased transfection efficiency suggesting cellular uptake of the complexes is through endocytosis. Although the study showed that lipofectin produced a slightly higher transfection

efficiency, the mPEG PLL copolymers were less cytotoxic and showed earlier gene expression. The 10 mole% copolymer showed the best transfection efficiency and the 25 mole% copolymer was considerably lower. The authors offer the explanation that this degree of mPEG grafting (25%) hinders contact of the complex with the cell membrane thus hindering penetration into the cell.

Recently, a PLL dendrimer has been synthesised and conjugated to mPEG-amine 6000 [Choi *et al*, 1999]. The PLL dendrimer, synthesised from L-lysine using Fmoc chemistry, has a globular structure with 16 surface amine groups. The study showed that this copolymer formed a water-soluble complex with DNA that increased the stability of the DNA to DNase I.

PEG-polyspermine block copolymer

A block copolymer of PEG 1500 and a polyamine with around 12 nitrogen atoms **91** (which the authors named *polyspermine*) has been prepared [Kabanov *et al*,1995]. The polyspermine was prepared by the reaction of 1,4-dibromobutane **89** with bis(3-aminopropyl)amine **88** followed by the reaction of this intermediate **90** with further 1,4-dibromobutane **89** (scheme **26**).



Scheme 26 Synthesis of *polyspermine* [Kabanov *et al*, 1995].

The product of the reaction in scheme **26** was a mixture of polyspermines and unreacted monomers. These were separated by gel filtration chromatography and the molecular weights determined by end group analysis; three fractions were obtained with molecular weights of 980, 720 and 490 g/mol. The 720Da polymer was conjugated to PEG 1500 using 1,1′-carbonydiimidazole as a coupling agent. This produced a mixture of triblock polymers PEG-polyspermine-PEG and polyspermine-PEG-polyspermine and the desired diblock PEG-polyspermine (figure **29**). To separate these, the authors conjugated the free amino groups to 2′-

deoxyadenosine, as a chromophore, and then carried out gel filtration followed by reverse-phase HPLC.

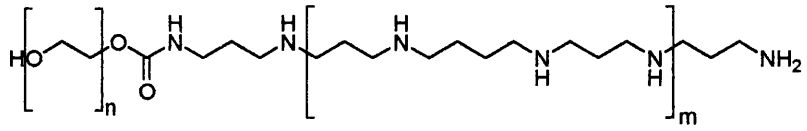
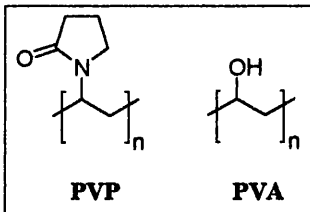


Figure 29 PEG-polyspermine block copolymer.

The formation of micellar complexes with an oligonucleotide was demonstrated by reverse-phase HPLC. The copolymer was shown to prolong the toxic effects of an antisense oligonucleotide against HSV-1 virus in Vero cells; the authors suggest that this is due to an enhanced uptake of the oligonucleotide and protection against nuclease degradation. A later study examined the properties of the complex formed between the PEG-polyspermine copolymer and phosphorothioate oligonucleotides [Vinogradov *et al*, 1998]. This study described the polyamine segment of the PEG-polyspermine copolymer as being randomly branched, this is not surprising given the method of synthesis. PEGylated PEI was also prepared using a similar coupling procedure. Both PEG-PEI and PEG-polyamine complexes with a phosphorothioate oligonucleotide were found to be stable at physiological pH and could be stored for several months after lyophilising. The complexes with PEG-polyamine were found to be *ca.* 12nm in diameter and the PEG-PEI, *ca.* 32nm, by light scattering and transmission electron microscopy techniques. The complexes were water soluble. Delivery of a phosphorothioate antisense oligonucleotide, targeted to fibronectin transcripts, to retinal vascular cells has been demonstrated using PEG-polyspermine as a carrier [Roy *et al*, 1999]. The oligonucleotide-polymer complex was injected intravitreally into rats; fibronectin mRNA levels were consistently decreased compared to a control. Fibronectin, a basement membrane protein, is over-expressed in diabetic retinopathy causing basement membrane thickening, a histological marker for this disease. This approach therefore has potential in diabetic retinopathy and may be useful in other retinal diseases.

Polyvinylpyrrolidone and polyvinyl alcohol

The neutral polymers polyvinylpyrrolidine (PVP) and polyvinyl alcohol (PVA), shown in figure **30**, were examined as carriers for gene delivery to skeletal muscle [Mumper *et al*,1996]. PVP is a strong hydrogen-bond acceptor, PVA functions as a hydrogen-bond donor. Formulations of pCMV-β-gal with PVP and with PVA were injected into rat tibialis muscle *in vivo* and resulted in an increase in the number and distribution of cells expressing β-gal compared to injections of the DNA formulated in saline. Up to a ten-fold enhancement in gene



PVP **PVA**

Figure 30 Polyvinylpyrrolidone and polyvinyl alcohol.

expression was observed over naked DNA using PVP of 50 kDa formulated at 5%, similar results were obtained with PVA 18 kDa formulated at 2%. Further studies were conducted to determine possible mechanisms of interaction of these polymers with DNA and the mechanism of enhancement of gene expression in muscle [Mumper *et al*, 1998]. Molecular modeling of PVP-DNA interaction showed that PVP forms hydrogen bonds with the base pairs of DNA within its major groove with the vinyl backbone of PVP providing a hydrophobic surface on the DNA. The addition of increasing amounts of PVP to DNA at pH 4 results in an increase in zeta potential corresponding to a decrease in the overall net negative charge. It was found that PVP carries a small positive charge in water below pH 5.5, but there is no evidence that PVP condenses DNA into particles as shown by laser light scattering of electron microscopy [Mumper *et al*, 1998]. Quenching of ethidium bromide-DNA complex fluorescence is observed with PVP in water at pH 4, a decrease in fluorescence of about 40% is seen at a mass ratio (PVP:DNA) of around 100. PVP and PVA delivery systems have been termed *Protective, Interactive, Non-Condensing (PINC)* systems [Mumper *et al*, 1998].

Considering the findings of the various *in vivo* delivery studies and the experiments to determine the mechanisms of interaction of PVP with DNA, formation of PVP-DNA complexes is thought to enhance expression of reporter genes in rat muscle *in vivo* by the following mechanisms [Mumper *et al*, 1996,1998; Mumper and Rolland, 1998]:

- Protection of plasmids from rapid nuclease degradation; this has been demonstrated *in vitro* using a DNase I degradation assay [Mumper *et al*, 1996]. This may be due to formation of a hydrophobic coating on the DNA.
- Dispersion of DNA throughout the muscle; this is likely to be due to osmotic effects.
- Retention of the intact DNA in muscle.
- Facilitation of the uptake of DNA by muscle cells, possibly due to increased hydrophobicity and decreased surface negative charge.

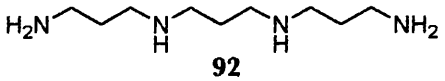
Recently, PVP has been used successfully to deliver the IL-12 gene to murine tumours *in vivo* [Mendiratta *et al*, 1999]. Intratumoural injection of the formulation led to IL-12 expression and subsequent induction of a potent antitumour immune response.

Chapter 5:

Synthesis of mPEG-Thermine Conjugates

1 Introduction

Recall that to date, gene therapy is severely limited by the lack of efficient gene delivery systems; all vectors that have been developed have significant drawbacks. It is becoming clear that agents that condense DNA, forming relatively insoluble particulate systems, have not lived up to early expectations since, though they can efficiently deliver DNA in cell cultures, they often have low transfection efficiency *in vivo*. There is therefore a need for new vectors. There has been increasing interest in water soluble complexes as opposed to lipidic systems [Kabanov *et al*, 1995; Toncheva *et al*, 1998; Wolfert *et al*, 1996; Choi *et al*, 1998; Mumper *et al*, 1996]. Broadly, the aim here was to synthesise a family of PEGylated polyamines that would be expected to form hydrophilic complexes with DNA capable of gene transfer *in vivo*. At the outset of the study, it was not certain how these conjugates would interact with DNA, whether they would condense DNA, forming a particulate system, or if the interaction would be somewhat weaker. This Chapter describes the synthesis of the first generation of these conjugates, those based on the linear tetramine, *thermine*‡ **92**. It will first be useful to consider, in general terms, how PEG may be conjugated to amines and how polyamines may be synthesised and protected.



92

1.1 Activation of PEG as an Electrophile

In order to react with amines, PEG must be activated in some way such that it is electrophilic. PEG electrophiles have been used extensively in the preparation of PEG-protein conjugates due to their reactivity towards lysine residues, which often provide convenient, unhindered sites for PEG attachment. Many PEG electrophiles have been developed [Zalipsky, 1995; Zalipsky and Lee, 1992] some examples are shown in figure **31**.

‡ The polyamine **92**, N,N′-bis(3-aminopropyl)propane-1,3-diamine/1,11-diamino-4,8-diazaundecane is a natural product isolated from the extreme thermophilic bacterium *Thermus thermophilus* and has been given the trivial name thermine [Oshima, 1975]; it is referred to as thermine throughout this thesis.

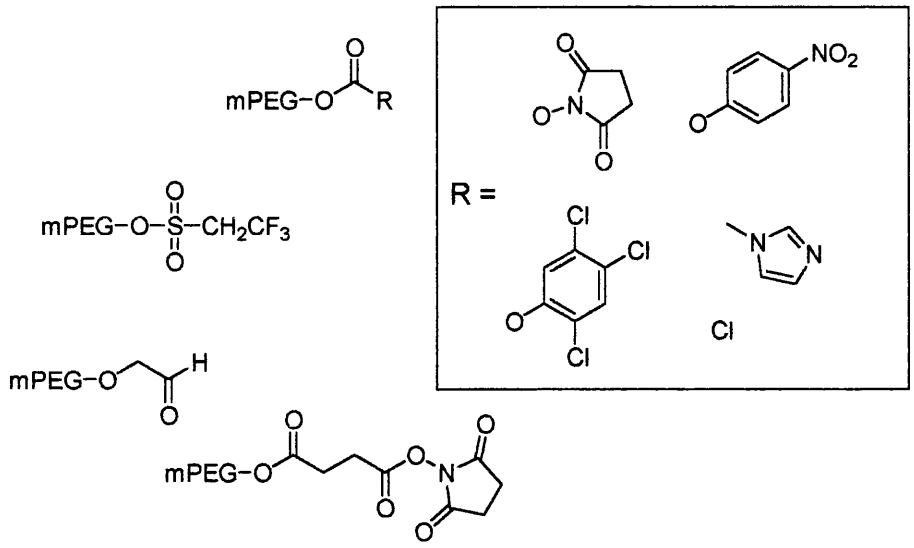
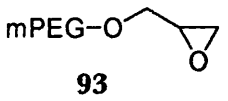


Figure 31 Electrophilically activated mPEGs.

The carbonate compounds are the most reactive towards amines and generate a carbamate linkage upon reaction. The tresylate is less reactive and is now thought to yield a sulfonate-amide upon reaction with amines [Gais and Ruppert, 1995]. The aldehyde is used under reductive conditions to yield a secondary amine, it has the advantage, in protein PEGylation, that its mild reactivity avoids potential side reactions with other nucleophilic functionalities. The acid chloride of mPEG 500 has been used to conjugate the mPEG to poly-L-lysine [Choi *et al*, 1998]. The initial approach to be used here however, is to use a PEG glycidyl ether (oxiranylmethyl ether) **93**.



93

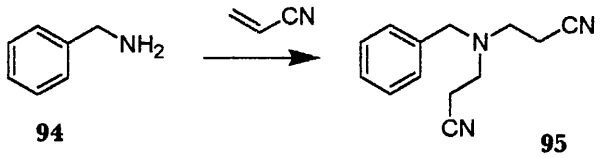
Why choose the glycidyl ether as the PEG electrophile?

- Reaction with a secondary amine forms a tertiary amine; reaction with a primary amine may form a secondary amine or a tertiary amine depending on the compound and conditions employed. Therefore the basicity is not quenched, as is the case for derivatives that form amides or carbamates, and thus the amine is able to be protonated.
- The pKa of the resulting secondary or tertiary amine may be such that it is only protonated within the endosomal compartment; this is potentially a desirable effect that is seen with polymers such as PEI and supposedly causes endosomal disruption aiding escape from the endosome.
- There is no leaving group, therefore there are less potential problems with purification of the polymer conjugate.
- Preparation of the PEG glycidyl ether is straightforward and it is relatively stable upon storage.

1.2 Synthesis of Polyamines

Cyanoethylation followed by reduction

The Michael-type addition of certain compounds to acrylonitrile which results in the formation of a molecule containing a cyanoethyl group is generally referred to as *cyanoethylation.* Many classes of compound undergo this type of reaction (including amines, certain amides, alcohols, thiols, oximes), most require catalysis, however amines add more readily than any other class of compound and do not generally require catalysis [Bruson, 1949]. A widely used procedure for the introduction of aminopropyl units is cyanoethylation followed by reduction of the nitrile. Primary amines may react with one or two moles of acrylonitrile; low temperatures favour the addition of one equivalent, high temperatures and excess acrylonitrile favour the addition of two equivalents [Bruson, 1949] and steric factors are also involved; the rate of addition to secondary amines decreases progressively with the size of the alkyl groups. Very harsh conditions are often required to force a primary amine to add twice to acrylonitrile. For example, the reaction of benzylamine **94** with acrylonitrile (scheme **27**) to yield the dicyanoethyl derivative **95** required heating to 110°C in a sealed tube for 7 days [Bergeron *et al*, 1981]



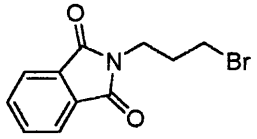
94 **95**

Scheme 27 Dicyanoethylation of benzylamine, used during the synthesis of bis(3-aminopropyl)amine.

Nitrile groups can be reduced to the corresponding amine with a variety of reagents, most commonly lithium aluminium hydride (LAH) or catalytic hydrogenation, typically over Raney nickel. Hydrogenation should be performed under basic conditions to suppress secondary amine formation by attack by the nascent amine on the imine intermediate; sodium hydroxide in EtOH [Bergeron and Garlich, 1984] or methanolic ammonia [Schlögl and Schlögl, 1964] are commonly used. Nitrile groups have been reduced, in the synthesis of a polyamine, using sodium borohydride in the presence of cobalt (II) chloride hexahydrate [Buhleier *et al*, 1978]; this procedure is essentially a catalytic hydrogenation as colloidal cobalt, catalytically analogous to Raney nickel, is formed *in situ* and hydrogen is evolved.

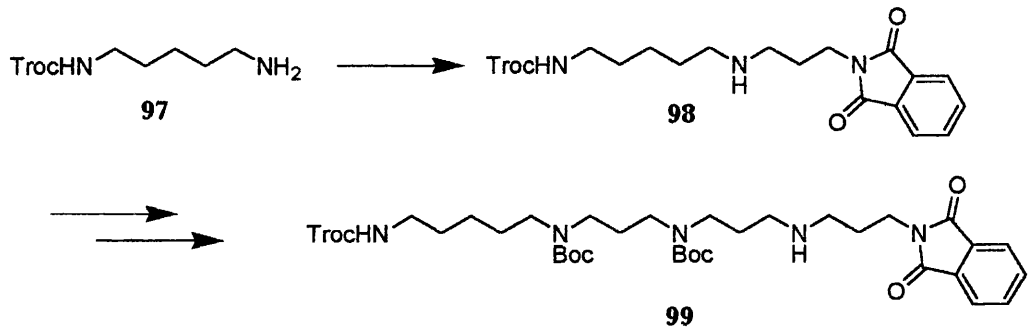
Alkylation of amines

Amines undergo alkylation by alkyl halides. Of particular use in polyamine synthesis is alkylation using bromoalkyl phthalimides such as N-(3-bromopropyl)phthalimide **96**. Bromoalkylphthalimides are



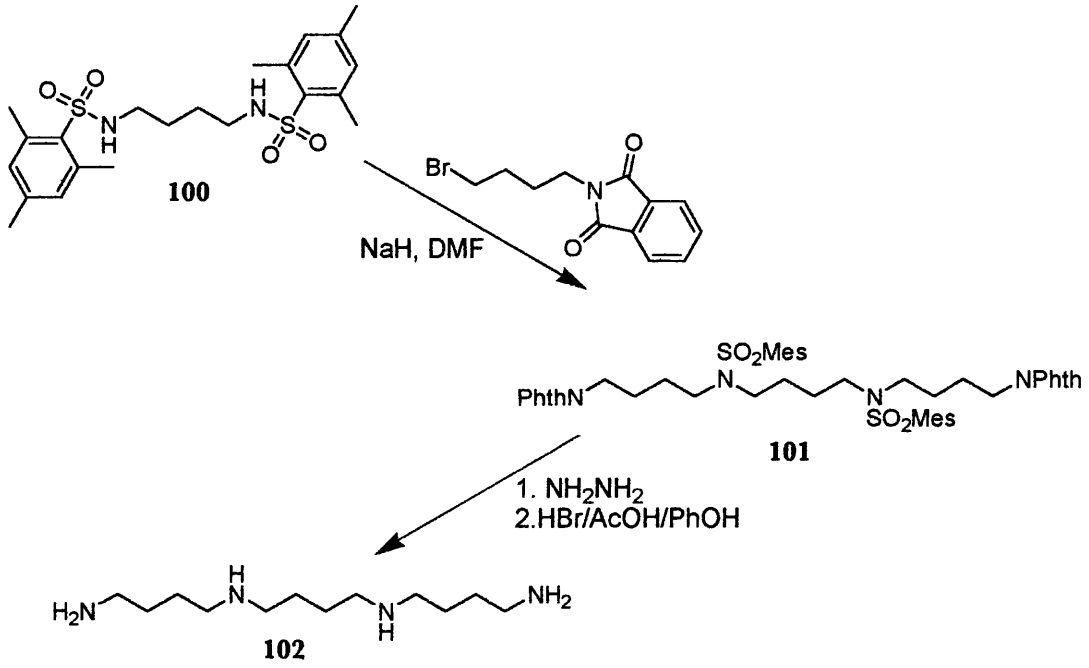
96

stable, readily available, cheap crystalline solids. The phthalimido protection on the amine can be removed efficiently using hydrazine. A typical example of the use of amine alkylation using phthalimides in polyamine synthesis is shown in scheme **28** [Jasys *et al*, 1992].



Scheme 28 An example of the use of N-(3-bromopropyl)phthalimide in polyamine synthesis.

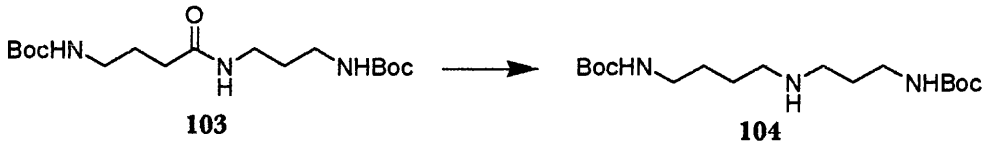
Compound **97** was treated with N-(3-bromopropyl)phthalimide **96** in the presence of KF on Celite in acetonitrile to yield **98**. The secondary amine was protected with Boc and the phthalimido group was removed with hydrazine. This sequence was repeated twice to yield tetra-protected pentamine **99**. KF on Celite is typically used in these reactions; it enhances the nucleophilicity of the alkylating species by hydrogen-bond formation with the fluoride anion [Ando and Yamawaki, 1979]. Formation of tertiary or quaternary amines presumably does not readily occur due to steric constraints. Alkylation can also be achieved by generating the anion of a sulfonamide (such as **100**) and allowing this to react with a halo compound, for example this approach has been used in the synthesis of homospermine **102**, scheme **29** [Bergeron *et al*, 1996].



Scheme 29 Synthesis of homospermine **102**.

Reduction of amides

Given the wealth of literature available concerned with peptide synthesis, *i.e.* making amides, it is perhaps surprising that the synthesis of polyamines *via* the reduction of amides has not been widely reported. This may be due to the harsh conditions that are often required to reduce amides, limiting functionality elsewhere in the molecule, and difficulties in isolating the highly polar polyamine products from borane or LAH reaction mixtures. A simple example of the use of this approach for the synthesis of diBoc spermidine **104**, by reduction of amide **103** using sodium triacetoxyborohydride, is shown in scheme **30** [Sundaramoorthi *et al*, 1984].

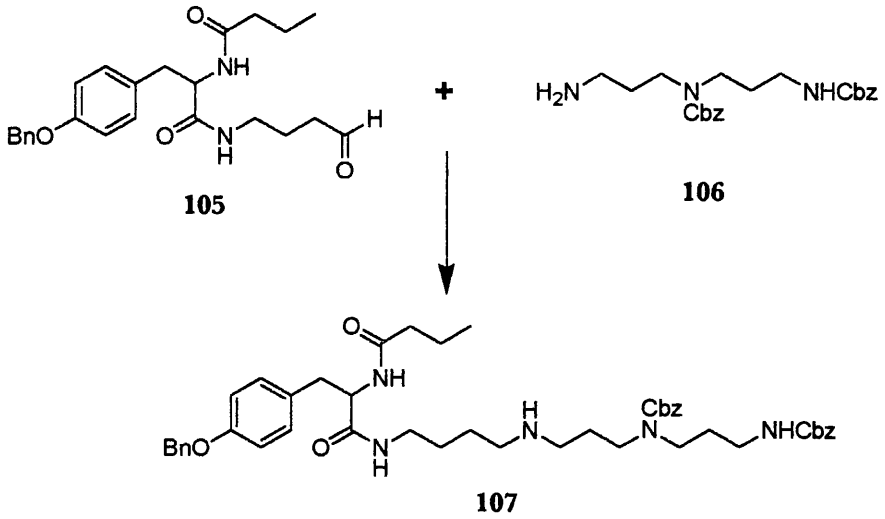


103 **104**

Scheme 30 Reduction of an amide in the synthesis of a spermidine derivative.

Reductive amination

Aldehydes react with primary and secondary amines to form imines; reduction of the imine *in situ*, typically with sodium cyanoborohydride, under mildly acidic conditions, gives the corresponding secondary or tertiary amine. This procedure is referred to as reductive amination or, alternatively, as reductive alkylation. Polyamines can be built with N-protected amino aldehydes using this procedure; amino aldehydes are easily synthesised from the corresponding amino alcohol by Swern oxidation. An example is illustrated in scheme **31** where a wasp toxin, philanthotoxin-4.3.3 (**107** after deprotection) was constructed using a reductive alkylation procedure [Moya and Blagbrough, 1995].



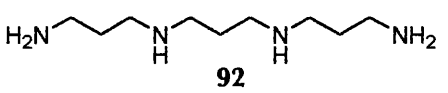
105 **106**

107

Scheme 31 Reductive amination in the synthesis of a polyamine derivative [Moya and Blagbrough, 1995].

1.3 Which Polyamines ?

Many studies using polyamines in the synthesis of gene delivery vectors or other applications have used the widely occurring polyamines such as spermine and spermidine. However, in this study it was decided to use thermine **92** as a *rational* starting point, where the methylene spacing between the nitrogens is 3-3-3 as opposed to 3-4-3 as in spermine.



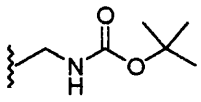
92

Furthermore, in a study on the compaction of DNA by diaminoalkanes, it was found that diaminoalkanes that possessed 3 or 5 methylenes between the nitrogens were considerably more efficient in inducing compaction than the other compounds (diaminoalkanes with between 1 and 6 methylenes were examined in the study) [Yoshikawa and Yoshikawa, 1995].

1.4 Protection of the Amino Group

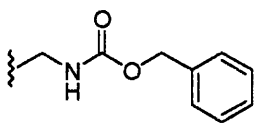
Polyamines possess many reactive nitrogens, thus an effective protection strategy is fundamental to the synthesis of polyamines and polyamine conjugates/analogues. Many amino protecting groups have been developed [Kocienski, 1994; Greene and Wuts, 1999] and much of the chemistry has come from the synthesis of protected amino acids for peptide synthesis. A brief account of the most commonly used amino protecting groups will follow.

t-Butoxycarbonyl (Boc)



The Boc group has been extensively applied in amine and amino acid protection chemistry. It is easily introduced with di-*t*-butyl dicarbonate (Boc_2O) [Tarbell *et al*, 1972] in a fast and high yielding reaction, the by-products being carbon dioxide and *t*-butanol. It is stable to many reaction conditions including catalytic hydrogenolysis, bases and nucleophilic reagents. It is easily and rapidly removed with acid, commonly HCl or trifluoroacetic acid. 2-(*t*-Butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON) can also be used to introduce Boc protection; it is less reactive than Boc_2O and has been applied to the selective protection of primary amines in the presence of secondary amines [Hu and Hesse, 1996].

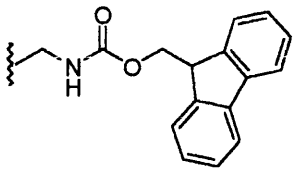
Benzyloxycarbonyl (Cbz)



The discovery of benzyloxycarbonyl protection in 1932 [Bergmann and Zervas, 1932] paved the way for significant advances in peptide synthesis and it remains a widely used, versatile protecting group. It is efficiently introduced to amino acids and other amino compounds under Schotten-Baumann conditions using benzyl chloroformate, although this reagent can be used to equal effect in organic solvent systems. It is highly stable in strongly basic conditions and acidic conditions up to pH 1. It is easily removed by catalytic hydrogenation typically with palladium on charcoal, often at

atmospheric pressure. HBr in glacial acetic acid or Na in liquid ammonia may also be used. Benzyl chloroformate has the disadvantage that it decomposes on storage, even at low temperature, and contents of up to 20% benzyl chloride can be observed [Wünsch, 1986] leading to possible side reactions. This can be avoided by the use of dibenzyl dicarbonate [Wünsch, 1986], although this reagent is expensive and requires storage at <0°C.

9-Fluorenylmethyloxycarbonyl (Fmoc)



This is introduced with 9-fluorenylmethyl chloroformate and can be removed selectively with a secondary amine such as piperidine. It is highly resistant towards acid, but may be sensitive to hydrogenolysis albeit at a much lower rate than Cbz. This group has been extensively applied to solution and solid phase peptide synthesis.

Boc, Cbz and Fmoc represent the most useful amino protecting groups and many protection strategies can be solved using these groups alone; all are fully orthogonal to each other. There are many other carbamate protecting groups including 2,2,2-trichloroethoxycarbonyl (Troc) and 2-(trimethylsilyl)ethoxycarbonyl (Teoc). The Troc group is useful as it is stable to acid and base (thus Boc and Fmoc can be removed in its presence) but is easily removed under mildly reductive conditions such as Zn/ammonium acetate which does not affect groups such as Boc or TFAc. Teoc can be removed using the highly selective reagents typically used to remove organosilicon protecting groups such as tetrabutylammonium fluoride.

Trifluoroacetyl (TFAc)

Amides are not generally useful as protecting groups due to the harsh conditions required to cleave them, the exception being trifluoroacetamide which is readily base hydrolysed and thus can be removed in the presence of Boc and Cbz. TFAc is a very useful amino protecting group as it can be introduced selectively to primary amines over secondary amines using ethyl trifluoroacetate [Xu *et al*, 1995]. Secondary amines can be protected with TFAc by reaction with trifluoroacetic anhydride.

Phthalimido (Phth)

The phthalimido group is useful as amines can be extended with aminoalkyl units using N-bromoalkyl phthalimides (see the discussion on polyamine synthesis above). Phth protection can be removed in high yield with hydrazine, conditions that do not affect Boc or Cbz.

Sulfonamides

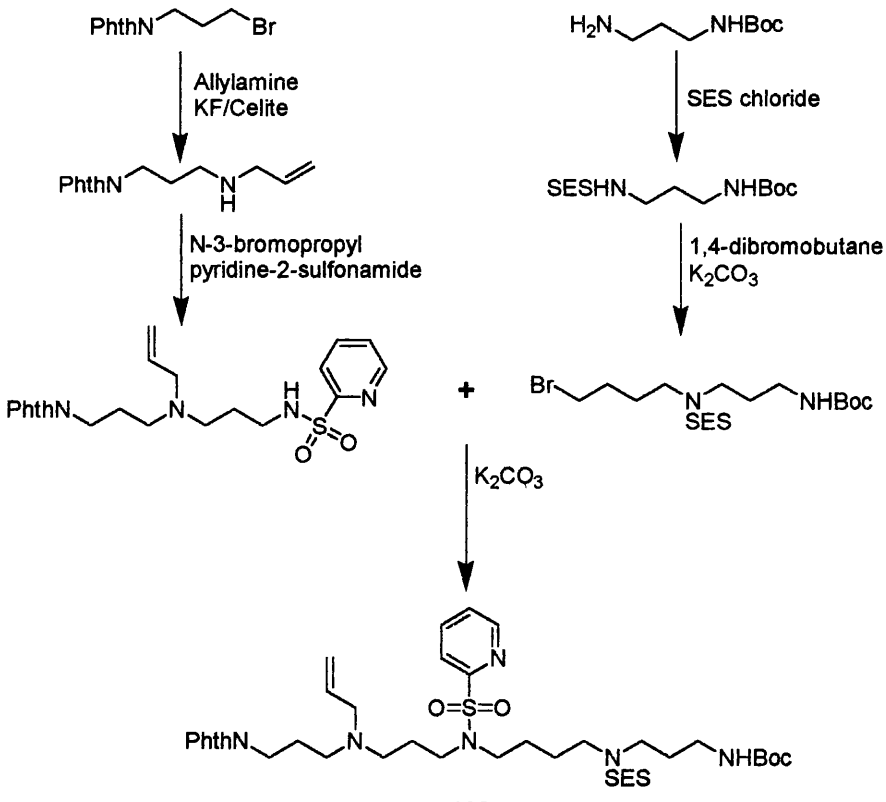
Sulfonamido protection is introduced using the corresponding sulfonyl chloride and can be removed using Na/liquid ammonia or HBr/glacial acetic acid/phenol. Examples of the use of sulfonamido protection in polyamine synthesis are provided in scheme **29** [Bergeron *et al*, 1996]

(page 86) and scheme **37** [Bergeron *et al*, 1988](page 95). Sulfonamides are very stable and often highly crystalline.

Benzyl

Although the benzyl group is not a particularly useful or often used protecting group for amines, as it is not very easily introduced or removed, it is worth a mention here because the di-functionalisation of benzylamine with cyano-alkyl chlorides or acrylonitrile (see scheme **27**) followed by reduction of the nitriles is a useful way into spermidine derivatives benzyl-protected on their central nitrogen [Bergeron *et al*, 1981]. Bergeron and co-workers were able to reduce nitrile groups without affecting the benzyl group using LAH [Bergeron *et al*, 1981]. N-Benzyl groups can be cleaved by catalytic hydrogenolysis, but the reaction is often slow and requires a higher catalyst loading and/or higher pressure of H_2 and/or a higher temperature then O-benzyl protection. Na in liquid ammonia provides an alternative, efficient removal of N-benzyl groups.

Recently Hesse and co-workers have described the construction of linear pentamines bearing five independently removable N-protecting groups [Pak *et al*, 1998; Pak and Hesse, 1998a,b] Scheme **32** shows some of their work [Pak *et al*, 1998] and serves to illustrate a good example of the synthesis/protection and deprotection of a linear pentamine.



108

Scheme 32 Synthesis of a penta-N-protected pentamine **108** with five independently removable protecting groups.

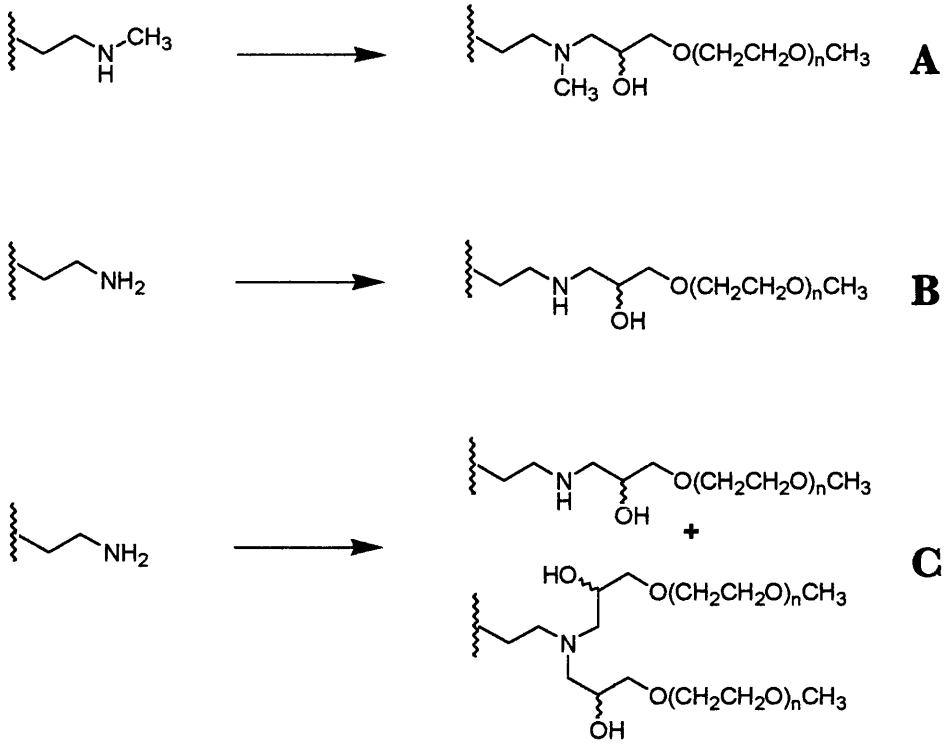
This represents an elegant five-step convergent synthesis of a penta-N-protected pentamine **108.** Each protecting group was removed independently thus allowing elaboration of any number of the five amines. The Boc group was removed with trifluoroacetic acid in dichloromethane. The triethylsilylethanesulfonyl (SES) group, which is stable to acid and base, was removed with CsF in DMF. The allyl group could be cleaved with N,N′-dimethylbarbituric acid and tetrakis(triphenylphosphine)palladium and the Phth with hydrazine. The authors found it was not possible to remove the 2-pyridinesulfonyl group (with SmI_2) without reduction of the Phth. They could however exchange the Phth group for TFAc (treatment with hydrazine followed by acylation with trifluoroacetic anhydride), this allowed the 2-pyridinesulfonyl group to be removed selectively and quantitatively by electrolysis. Finally the trifluoroacetyl group could be hydrolysed with potassium carbonate in $MeOH/H_2O$.

1.5 Aims and objectives of this work

- To explore established methods and/or to develop new methods for the synthesis of a range of suitably protected polyamines, for coupling to electrophilically activated mPEGs.

- To synthesise mPEG derivatives, suitably activated to allow coupling to primary and or secondary amino groups. Specifically, it was intended to utilise mPEG glycidyl ethers.

- To investigate the reaction of PEG glycidyl ethers with suitably protected polyamines. This should determine whether or not it is desirable to N-methylate the amine (scheme **33,** line **A**); or, whether or not the reaction can be controlled such that the PEG glycidyl ether adds only once to a primary amine (scheme **33,** line **B**); or, whether or not it is possible to separate mono from diPEGylated material (scheme **33,** line **C**) using the methods available on a large scale *i.e.* column chromatography on silica. It was intended to synthesise symmetrical diPEGylated and unsymmetrical monoPEGylated conjugates of thermine, and PEG conjugates of branched polyamines.



Scheme 33 Possible reactions of mPEG glycidyl ethers with amines.

- To measure the ability of the synthesised conjugates to form complexes with DNA, using a spectrofluorimetric assay, in order to establish their potential as excipients for the formulation of gene medicines.

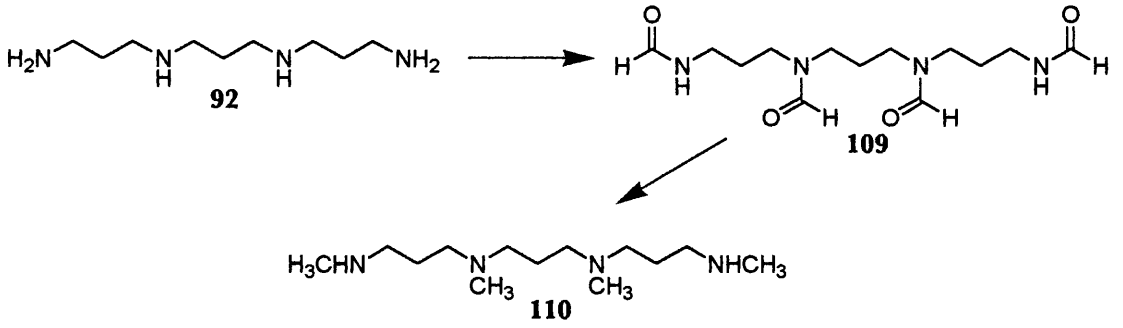
2 Synthesis of Thermine Derivatives for Coupling to mPEG

At the outset of this study it was felt that separation of mixtures of different PEGylated polyamines by chromatography may not be practicable and that coupling using secondary amines would be desirable; thus initially N-methylated thermine derivatives were prepared. In practice it was possible to explore coupling to both primary and secondary amines with success and both methods have advantages and disadvantages.

2.1 N^1,N^5,N^9,N^{13}-Tetramethylthermine

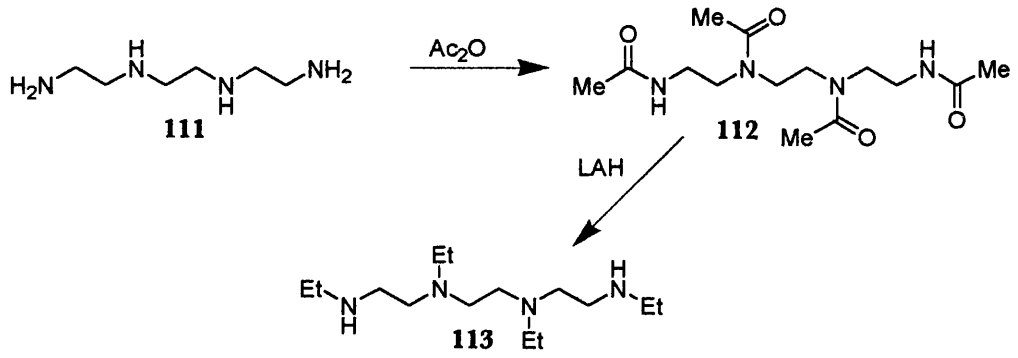
Route attempted via N^1,N^5,N^9,N^{13} tetraformylthermine

The simplest thermine derivative that could be coupled to an electrophilically activated mPEG, such as the glycidyl ether, to form a symmetrical α,ω-diPEGylated conjugate, is the tetramethyl compound **110** (scheme **34**). This would require no modification, such as protecting group removal, after conjugation. This was the first target polyamine. It was thought that the methyl group would not interfere with the electrostatic interaction of the protonated amine with DNA.



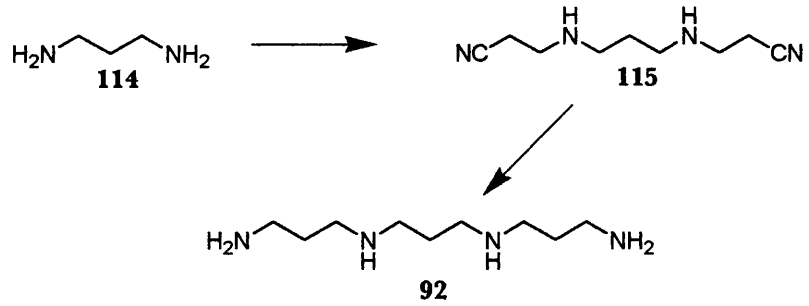
Scheme 34 Proposed synthesis of **110**.

The proposed synthesis was to make the tetraformyl derivative **109** of thermine **92** and then to reduce the amides to give the tetramethyl compound **110**. Krakowiak *et al* [Krakowiak *et al*, 1989] described the synthesis of the tetra-N-ethyl homologue of **111** (scheme **35**) by reducing the tetraacetamide **112** with LAH.



Scheme 35 Synthesis of a tetraethyl polyamine **113** by reduction of the tetraacetamide **112** [Krakowiak *et al*, 1989].

Further, formylation followed by reduction using $BH_3.Me_2S$ has been described as a general procedure for the production of N-methyl (N-alkyl) amines [Krishnamurthy, 1982] and, in general, the reduction of primary, secondary and tertiary amides to yield their corresponding amines is a well-documented procedure [Brown and Heim, 1973; Brown *et al*, 1981a,b]. Treatment of thermine with excess ethyl formate gave tetraformamide **109** in 85% yield after chromatography. Although thermine is commercially available, the commercial material is expensive and of variable quality. Thermine **92** was easily prepared by the symmetrical dicyanoethylation of propane-1,3-diamine **114**, in quantitative yield by treatment with two equivalents of acrylonitrile at 0°C then room temperature, followed by reduction of dinitrile compound **115** by catalytic hydrogenation over Raney nickel in methanolic ammonia (scheme **36**).



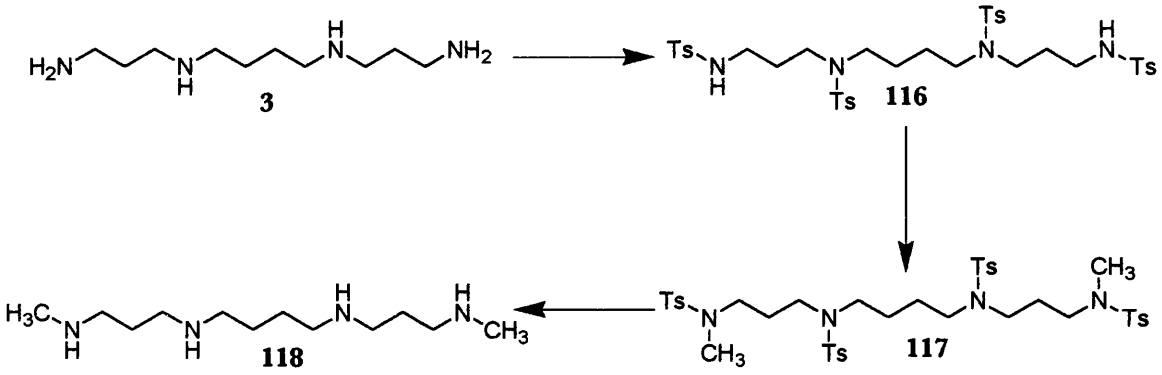
Scheme 36 Preparation of thermine **92**.

After filtering off the catalyst, washing it, and evaporating the methanolic ammonia, the sample appears as a blue oil. This is thought to be due to a complex forming between trace amounts of Ni^{2+} ions and the polyamine or ammonia or involving both. This did not pose a purification problem in this case, the thermine was obtained as a colourless liquid in 67% yield by Kugelrohr distillation of the crude, blue material.

The tetraformamide was found to have very low solubility in a range of organic solvents. It was insoluble in the aprotic solvents commonly used for LAH or borane reductions, such as diethyl ether, THF, dioxane or 1,2-dimethoxyethane. It was soluble in bis(2-methoxyethyl)ether (diglyme) at temperatures near its boiling point (162°C) and was slightly soluble in CH_2Cl_2. Reduction was attempted using LAH in boiling diglyme; however, numerous new products were observed by TLC. The reduction was attempted using $BH_3.Me_2S$ in CH_2Cl_2. Although the tetraamide was only slightly soluble in CH_2Cl_2, it was thought that the product would have a higher solubility. Hence, as product forms, more tetraamide may go into solution and eventually the reaction may go to completion. Thus the reduction was attempted using the method of Brown [Brown *et al*, 1981a,b]. The amide was treated with $BH_3.Me_2S$ in CH_2Cl_2 in the presence of $BF_3.Et_2O$. No products could be isolated from the reaction mixture. In conclusion, reduction of tetraformamide **109** is not a viable method for the synthesis of tetramethyl amine **110**, thus an alternative method was sought.

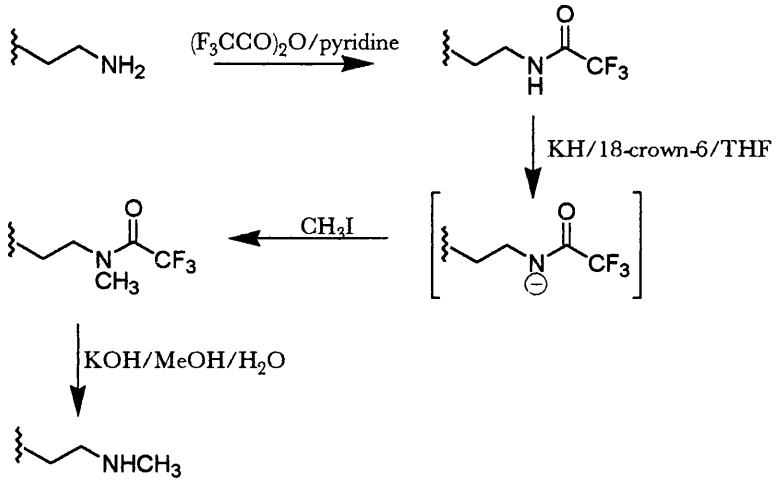
Route attempted via N^1,N^{13}-bis(trifluoroacetyl)-N^5,N^9-dimethylthermine

The terminal primary amines of spermine have been N-methylated (scheme **37**) by preparation of tetratosyl spermine **116** followed by treatment with sodium hydride and methyl iodide, then removal of the tosyl protection with Na/liquid ammonia [Bergeron *et al*, 1988].



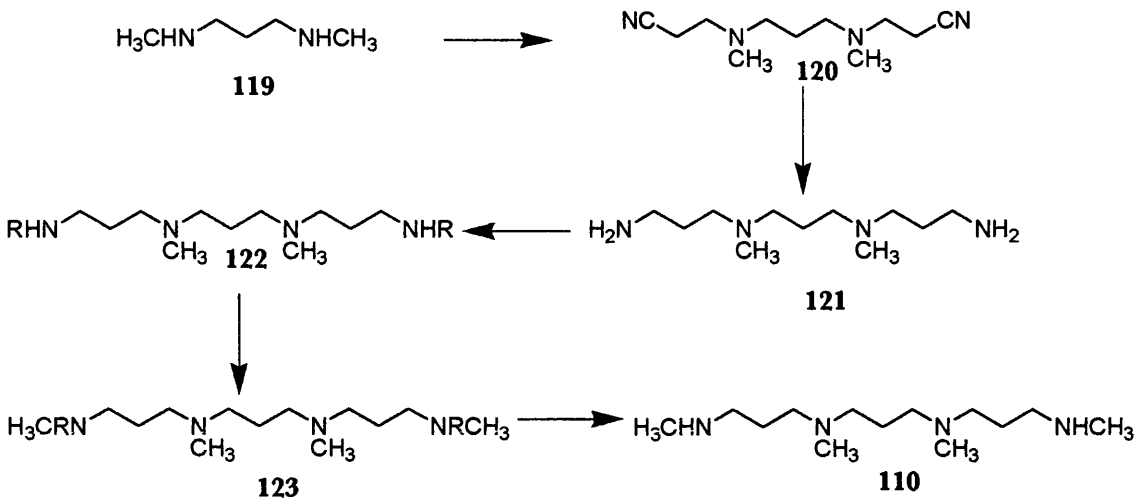
Scheme 37 Preparation of dimethylated spermine from spermine **3** [Bergeron *et al*, 1988]

Trifluoroacetamide derivatives have also been used in this way for N-methylation; an example is illustrated in scheme **38** [Broka and Gerlits, 1988].



Scheme 38 Methylation of a primary amine *via* its trifluoroacetamide [Broka and Gerlits, 1988].

Obviously, this type of method could not yield the required tetramethyl compound **107**, as secondary amines cannot be methylated in this way. However, the diamine, N,N′-dimethylpropane-1,3-diamine, **119** (scheme **39**), is commercially available and could thus be used as a starting material. Following the successful cyanoethylation and reduction sequence used in the synthesis of thermine, an analogous procedure utilising the dimethylated diamine **119** would yield **121**. This could be N-methylated using a similar procedure to that used for the synthesis of dimethylated spermine **118**. The reduction of **120** by hydrogenation over Raney nickel had previously been reported [Schlögl and Schlögl, 1964].



Scheme 39 Proposed synthesis of tetramethyl amine **110**; R would be tosyl or trifluoroacetyl.

Thus **119** was treated with acrylonitrile to yield the dinitrile **120**, quantitatively. Compound **120** was reduced with H_2/Raney nickel in saturated ethanolic NaOH at 35°C. NaOH was used as the base to investigate if this would suppress the formation of the nickel complex seen when methanolic ammonia is used. After the catalyst was removed, the mixture was concentrated to low volume and the amine was caused to come out of solution by the addition of 70-80% aqueous NaOH. Extraction of the highly polar amine with chloroform proved to be difficult due to formation of emulsions. However, the crude amine was indeed colourless prior to Kugelrohr distillation and not a blue nickel complex. The tetramine **121** was obtained in 36% yield. It is likely that the low yield is largely due to poor recovery of the polyamine from the NaOH mixture. In a further attempt to improve the yield, the reaction was carried out in methanolic ammonia as described for the synthesis of thermine. This again gave a blue product. This time however, the Ni^{2+} contaminant was removed by precipitation as NiS, by bubbling H_2S through a solution of the amine. This gave the crude amine as a pale brown oil. Attempts to purify this by making the hydrochloride salt and then recrystallising it were unsuccessful. However, the ^1H NMR spectrum illustrated that the material was of sufficient purity to be useful synthetically and is shown in figure **32**.

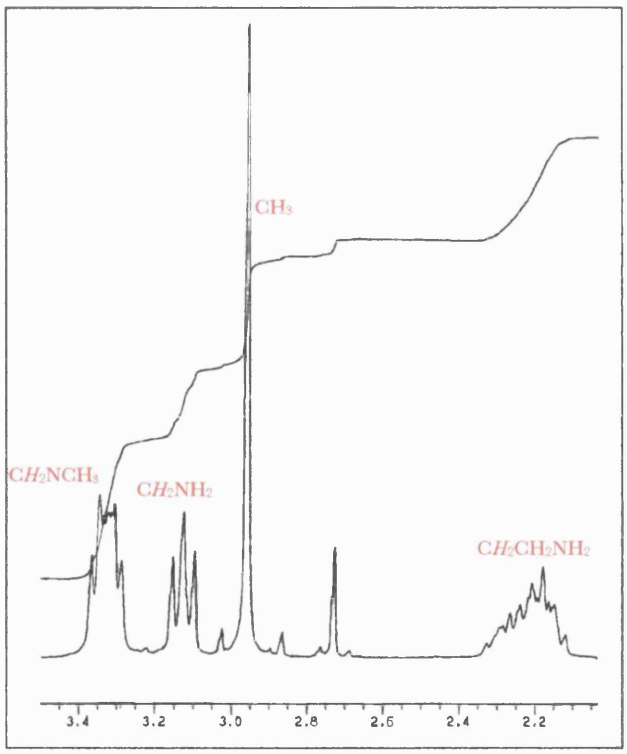
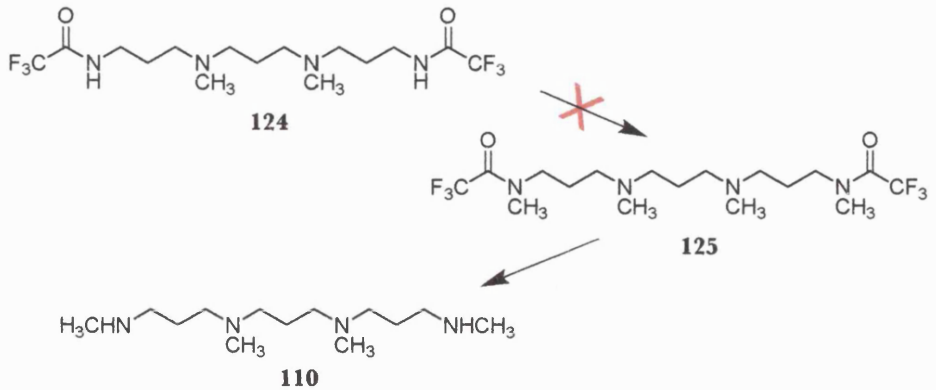


Figure 32 ^{1}H NMR spectrum of the hydrochloride salt of compound **121** (270MHz, D_2O).

Following the successful synthesis of the tetramine with methyl groups on the middle secondary nitrogens, attempts could be made to methylate the terminal primary amino groups. It was decided to use the bis(trifluoroacetamido) derivative (scheme **40**) as the intermediate for the methylated tetramine as this group is easier to introduce, and also to remove, than tosyl groups, cleavage of which requires harsh procedures using Na/liquid ammonia or HBr/glacial acetic acid. Hence, the bis(trifluoroacetamide) **124** was prepared in quantitative yield by treatment of **121** with ethyl trifluoroacetate in MeOH at room temperature. The use of ethyl trifluoroacetate provides a mild and efficient introduction of the TFAc group to primary amines; the reaction by-product following acyl transfer, EtOH, is simply evaporated from the reaction mixture along with any excess reagent [Xu *et al*, 1995; Curphey, 1979].

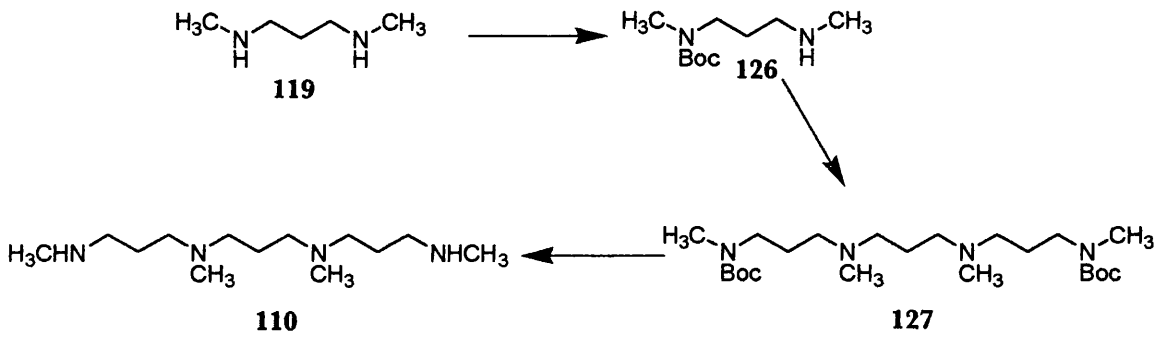


Scheme 40 Proposed use of bis(trifluoroacetamide) **124** as an intermediate in the synthesis of N^1,N^5,N^9,N^{13}-tetramethylthermine.

Attempts to methylate compound **124** failed. The bis(trifluoroacetamide) was treated with potassium *tert*-butoxide in dry THF under nitrogen, followed by methyl iodide. Although there was some evidence of further methyl groups in the ^{1}H NMR spectrum of the crude product, recovery of organic products, after aqueous work up to remove the inorganic by-products, was low. There are were other problems associated with this reaction. Firstly, the trifluoroacetamide is a hygroscopic oil and it is difficult to remove all traces of water from it, this may react with the potassium *tert*-butoxide and/or the formed anion. Secondly, there is a chance some of the methyl iodide may react with the middle tertiary amines forming quaternary ammonium salts. In addition, the di-anion is not very soluble in THF. A third synthesis was proposed, this proved to be successful and is described below.

Route from t-Butyl N-methyl-N-(3-methylaminopropyl)carbamate

In this approach, no methyl groups are introduced as electrophiles, rather the molecule is constructed by joining two N,N′-dimethylpropane-1,3-diamine molecules together to form the tetramine. The approach is illustrated in scheme **41**.

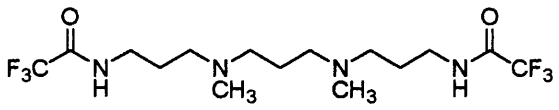


Scheme 41 Synthesis of tetramethylthermine **110** from N,N′-dimethylpropane-1,3-diamine **119**.

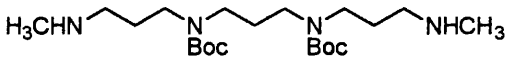
N,N′-Dimethylpropane-1,3-diamine **119** was treated with $^1/_3$ equivalents of di-*tert*-butyl dicarbonate in THF to give the mono-Boc derivative **126** in 71% yield after chromatography. Treatment of **126** with ½ equivalents of 1,3-dibromopropane in DMF at 80°C gave the desired tetramethyl compound as the diBoc derivative **127** in 52% yield after chromatography. The Boc protection was removed by treatment with 5M hydrochloric acid to yield the tetramethyl compound **110** as its tetrahydrochloride salt.

2.2 N^5,N^9-diBoc-N^1,N^{13}-dimethylthermine

Recall that the methylation of **124** was unsuccessful, possibly due to poor extraction of the product during aqueous work up or to low solubility of the di-anion in THF.



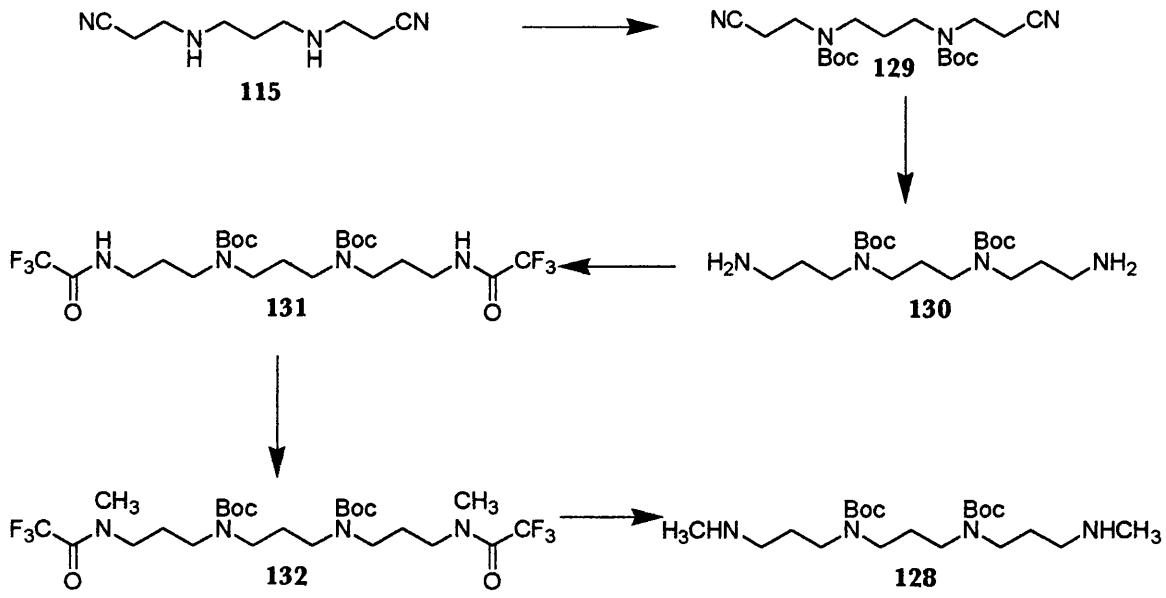
124



128

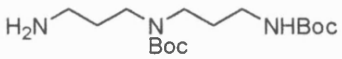
An approach to overcome these problems was to replace the middle methyl groups with a protecting group that would increase the solubility of the compound in organic solvents to make extraction from inorganic by-products easier and increase the solubility of the di-anion in THF. It is important that the protecting group should be easy to remove, once coupling to PEG had been achieved. The Boc protecting group is easy to introduce, should be stable under the PEG coupling reaction conditions and is easy to remove with acid, generating only gaseous by-products. This group should improve the solubility of the tetramine in organic solvents and the solubility of the di-anion in THF and was the protecting group of choice. Compound **128** was thus the target polyamine for coupling to mPEG and the proposed synthesis is shown in scheme **42**. Synthesis of this protected polyamine was also desirable because its mPEG conjugates may have potential advantages; the less polar nature of this protected polyamine may make purification and characterisation easier and the absence of methyl groups on the middle nitrogens makes hindrance of DNA interaction less likely.

The preparation of diBoc protected tetramine **130** by symmetrical dicyanoethylation of propane-1,3-diamine (as in scheme **34**) had been described by McCormick *et al* [McCormick *et al*,1993]. Treatment of **115** with Boc_2O gave **129**, reduction with LAH gave the diamine **130**.



Scheme 42 Synthesis of compound **128**.

The procedure was carried out according to the literature procedure [McCormick *et al*, 1993]. The dinitrile **115**, used in the preparation of thermine (scheme **34**), was treated with Boc_2O in CH_2Cl_2 to give the diBoc protected derivative **129** in quantitative yield. Reduction of the nitrile groups was carried out using LAH in dry ether/THF at 0°C. The reduction step, however, gave only 22% yield of the desired diamine, the reaction yielding a significant quantity of a by-product **133**. This presumably formed *via* a reverse Michael addition type mechanism.



133

Indeed, McCormick *et al* [McCormick *et al*, 1993] did report the reaction to be of disappointing yield:

.....The one disadvantage of this route is the disappointing yield (40 to 50%) in the nitrile reduction. The reduction was also carried out via catalytic hydrogenation (Adams' catalyst, Raney Nickel and Pearlman's catalyst), but none of these reactions represented an improvement.

In contrast to the findings of McCormick *et al*, reduction of **129** by catalytic hydrogenation improved the yield to 69%. Compound **129** was treated with Raney nickel in methanolic ammonia under 50psi of hydrogen for 72 hours. This yielded a blue oil which was purified by column chromatography. The terminal free amino groups were converted to their trifluoroacetamide derivatives by treatment with ethyl trifluoroacetate in MeOH at room temperature. Treatment of **131** with potassium *t*-butoxide, followed by methyl iodide in dry THF gave the methylated amide **132** which could be isolated by a conventional work up; evaporation of the THF left a residue which was dissolved in CH_2CH_2 and was washed with water to remove the inorganic by-products. The yield of the reaction was disappointingly low, until freshly distilled THF was employed when highly pure **132** could be isolated in 98% yield without the need for chromatography. Initially it was not obvious from the ^1H NMR spectrum that methylation had occurred. However, careful analysis suggested the new methyl signal appeared as two peaks due to rotational isomerism. Re-running the spectrum at 100°C overcomes the barrier to rotation and the methyl signal appears as one peak. Interestingly, the ^{19}F spectrum (figure **33**) shows two peaks in a 2:1 ratio suggesting the ratio of the *Z* to the *E* rotational isomers is 2:1. The trifluoroacetamides were effectively cleaved by treatment with concentrated aqueous ammonia in methanol (1:4) in a sealed vessel at 55-60°C. This gave the dimethylated diBoc-protected tetramine **128** in 79% yield after chromaography.

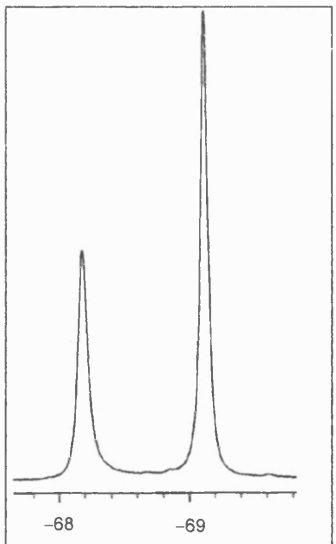


Figure 33 ^{19}F spectrum of compound **131**.

Compound 131 can be synthesised by an alternative method

Note that the method used to introduce the trifluoroacetamido group, an acyl transfer using ethyl trifluoroacetate, was that described by Xu *et al* [Xu *et al*, 1995]. In this communication, the authors describe how this reagent can be used to differentiate primary from secondary amino functions. Using this methodology it was possible to selectively trifluoroacetylate the terminal primary amino groups of thermine. Thermine was treated with 2 equivalents of ethyl trifluoroacetate in MeOH at 0°C then room temperature. Simply evaporating the solvents gave highly pure bis(trifluoroacetamido) compound **134** in quantitative yield (scheme **43**). The ^{13}C NMR of this product shows a good example of fluorine-carbon coupling and this is illustrated in figure **34**.

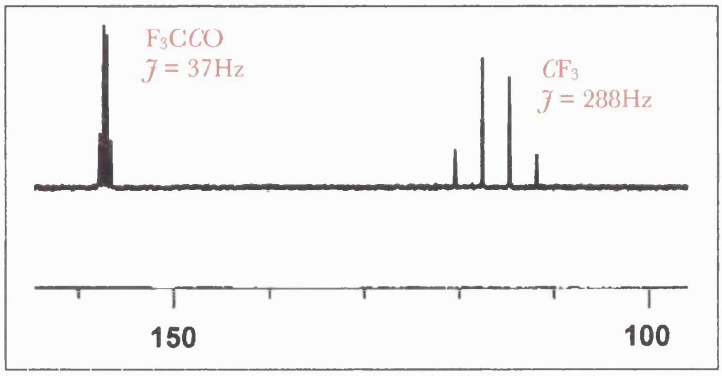
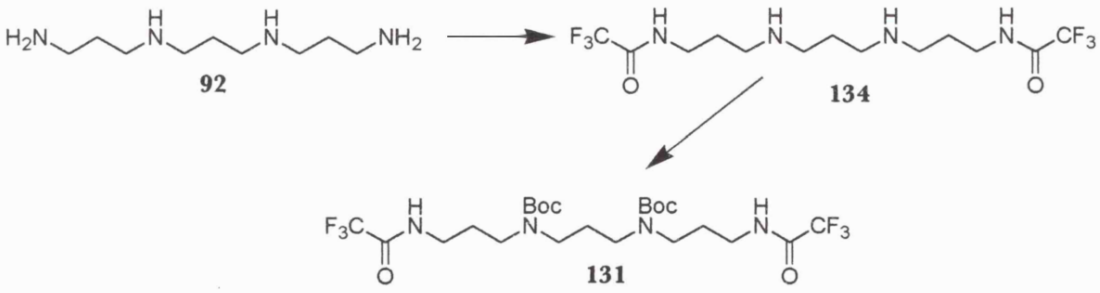


Figure 34 section of the ^{13}C spectrum of compound **134** illustrating fluorine-carbon coupling.

Treatment of **134** with di-*t*-butyl dicarbonate in THF at 0°C, then room temperature gave the bis(trifluoroacetamido)-diBoc compound **131** in quantitative yield.



Scheme 43 Synthesis of compound **131** from thermine.

This therefore represents an alternative route to the fully protected intermediate **131** in two steps (quantitative yield) from thermine **92** as opposed to four steps (69% yield) from propane1,3-diamine (scheme **42**).

3 Coupling of mPEG to the polyamines

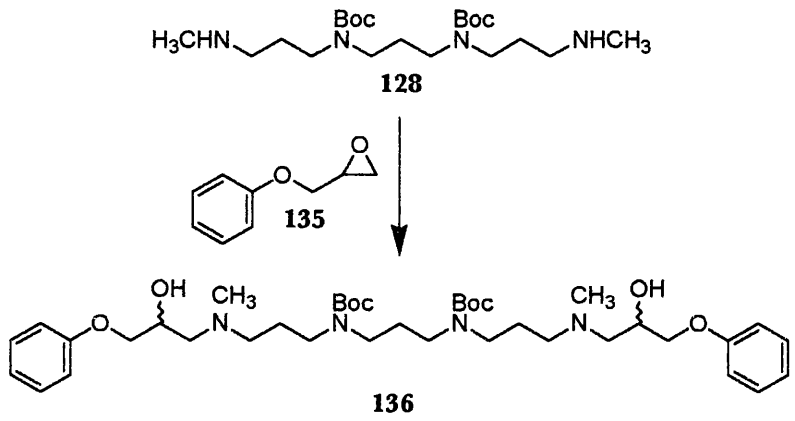
3.1 Overview

When deciding which mPEG molecular weight would be desirable for conjugation to the polyamine it was considered that using a PEG of molecular weight greater than about 4000Da may hinder binding of the polyamine to the DNA and would also make chemical characterisation of the conjugate more difficult. The molecular weight would however need to be higher than about 400Da to avoid potential future problems with toxicity. A sample of mPEG 2000 glycidyl ether was available‡ , so it was decided to use this as a starting point.

The reaction between epoxides and amines has been widely studied, largely because of its use in the curing of epoxy resins. The reaction occurs most readily in hydroxylic solvents [Shechter *et al*, 1956] and there is no great difference in the reaction of primary amines compared to that of secondary amines; sterically hindered amines react more slowly. The study by Shechter *et al* [Shechter *et al*, 1956] suggests that the possible competing reaction, that between hydroxyl group, either from the solvent or the ring opened epoxide, and epoxide does not occur to a noticeable extent. The choice of hydroxylic solvent here was *iso*propanol, being less nucleophilic than lower alcohols and allowing a higher reaction temperature if required.

3.2 Preliminary Reaction with Phenyl Glycidyl Ether

Before using the mPEG glycidyl ether, the reaction was tried using phenyl glycidyl ether **135** as a model. It was thought that this would give a product that was easy to purify and characterise and thus demonstrate the feasibility of the coupling reaction. The diBoc-protected polyamine **128** was used as the resulting construct may be easier to purify.

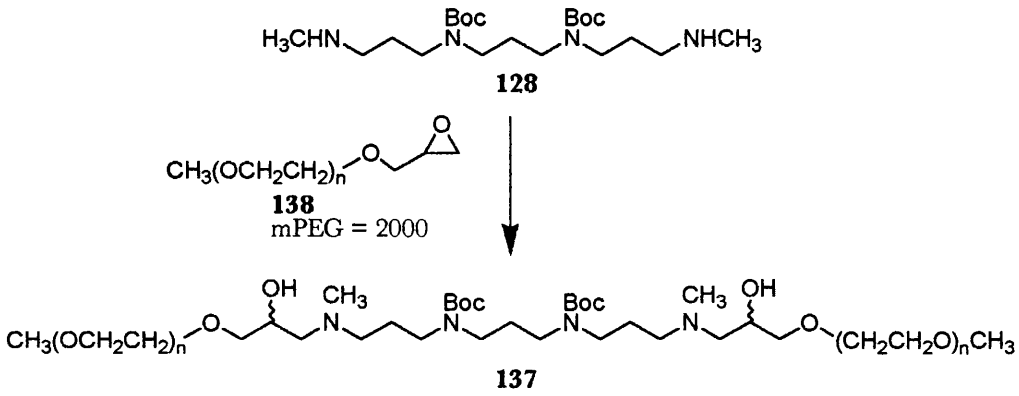


Scheme 44 Model glycidyl ether coupling reaction.

‡ I am grateful to Dr S.E. Matthews, Institute of Macromolecular Chemistry, Prague, Czech Republic for the kind gift of a sample of mPEG 2000 glycidyl ether.

Thus compound **128** was treated with 2 equivalents of phenyl glycidyl **135** ether in *iso*propanol (scheme **44**) at reflux for 10 hours. The product **136** was isolated by preparative TLC in 26% yield. The low yield may have been due to poor recovery of product from the crude mixture by the preparative TLC method; the reaction may also require longer than 10 hours to proceed fully.

3.3 Coupling with mPEG 2000 Glycidyl Ether



Scheme 45 Synthesis of mPEG 2000 conjugate of **128**.

Again the diBoc-protected polyamine **128** was used, this time it was felt the Boc methyl signals may provide a useful ^{1}H NMR reference. A solution of the polyamine **128** and two equivalents of mPEG 2000 glycidyl ether **138** in *iso*propanol was heated to reflux for 27 hours. A minor difficulty was monitoring the progress of the reaction; it was difficult to visualise the conjugate on TLC. Reaction progress could be followed by observation of a fall in intensity of the spot corresponding to the starting amine. Although the evidence from TLC indicated that a conjugate had formed, confirmation that the mPEG epoxide had indeed reacted with each amine was required. It was not possible to obtain a mass spectrum with the instrument available and it could not be decided with certainty from the ^{1}H NMR spectrum that conjugation had occurred. The peaks corresponding to the protons of the polyamine chain, and the atoms linking the PEG to the polyamine, were very small in comparison to the peak for the methylenes of the mPEG chain. ^{1}H NMR decoupling experiments, involving signals that potentially corresponded to the protons of 2-hydroxypropyl unit linking the PEG and amine, provided some evidence, albeit weak, that the coupling had been successful. A matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrum was required to confirm that the diPEGylated conjugate **137** had been synthesised, the spectrum is shown in figure **35**.

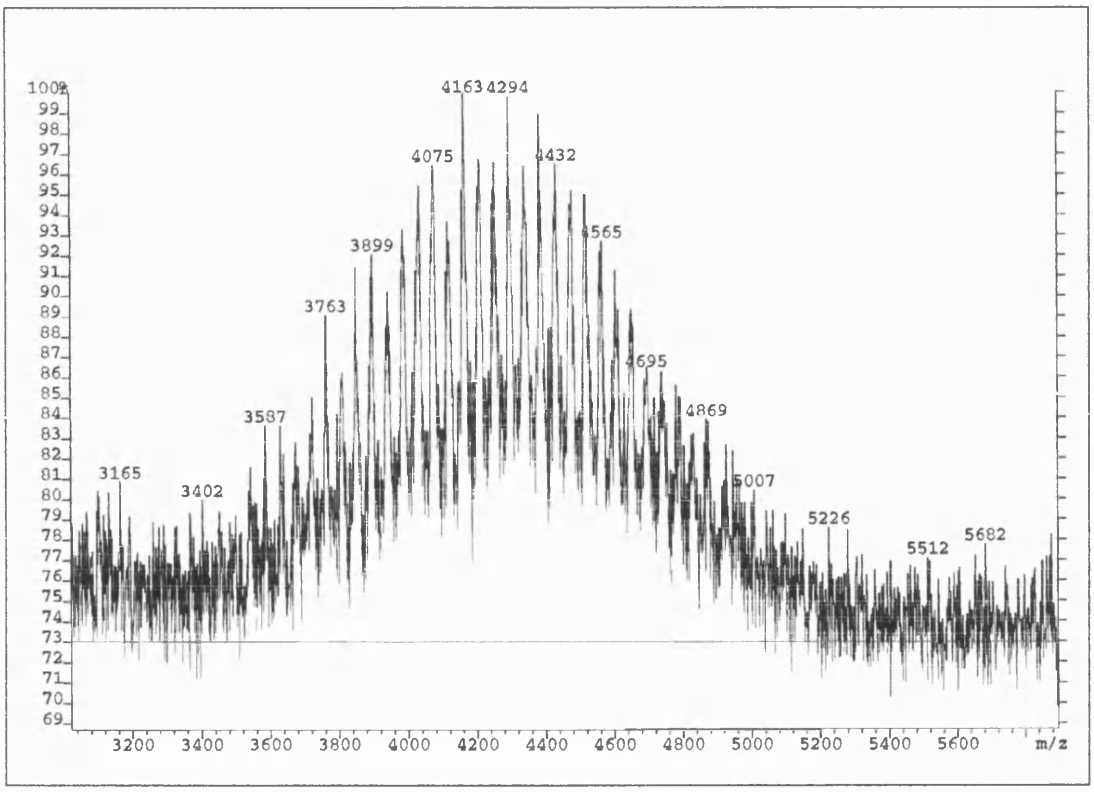


Figure 35 MALDI-TOF spectrum of conjugate **137**.

3.4 Coupling with mPEG 550 Glycidyl Ether

Following the difficulties in characterising the mPEG 2000 conjugate, it was decided to use a lower molecular weight mPEG (550Da), so that NMR spectra may be clearer and it may be possible to gain useful mass spectra, as the molecular weight of the conjugates should be within the capabilities of the available instrument using FAB or electrospray ionisation.

Synthesis of mPEG 550 glycidyl ether

The method used here was adapted from the method of Gu *et al* [Gu *et al*, 1985; Matthews, 1995] for the synthesis of diglycidyl ethers of oligo(ethylene glycol)s using epichlorohydrin and sodium hydroxide under phase-transfer catalysis. The work of Gu *et al* describes the effect of phase transfer catalysis and molecular weight of the glycol upon the efficiency of the reaction. The reaction proceeded more efficiently, even in the absence of catalysis, with increasing molecular weight and the authors suggest this is due to increased solubility of the alkoxide anion in epichlorohydrin. Thus, it was reasonable to assume that using mPEG 550 may require no catalysis. mPEG 550 was dried by azeotropic removal of water in toluene and then treated with epichlorohydrin, NaOH and water at 60°C for 3 hours. A small amount of water was added to allow a greater amount of NaOH into solution. The reaction solvent is effectively epichlorohydrin as a large excess is used (12eq). After the reaction the mixture is dissolved in

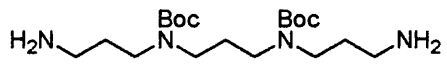
CH_2Cl_2 and the NaOH filtered off. The mixture requires evaporation under high vacuum for several hours to remove all the excess epichlorohydrin. Complete conversion of the hydroxyl to glycidyl ether could be conveniently determined by ^1H NMR in $[^2H]_6$-DMSO. The mPEG OH resonates as a clear triplet at δ4.56 [Dust *et al*, 1990], disappearance of this signal indicates complete conversion to the glycidyl ether.

Coupling

Compound **128** was treated with mPEG 550 glycidyl ether in *iso*propanol at reflux for 29 hours. This time, however, it was possible to obtain a FAB mass spectrum of the product which confirmed that the diPEGylated conjugate had been synthesised. The mass spectrum shows the typical distribution of peaks, 44 mass units apart, expected for the mPEG 550. The reaction was repeated with the tetramethyl compound **110** and mPEG 550 glycidyl ether in *iso*propanol. The free amines were generated by the addition of a slight excess of 5M aqueous NaOH prior to the addition of the glycidyl ether. One question that did arise at this stage was of the purity of these conjugates. By TLC (the mPEG 550 conjugates were easier to see on TLC than the mPEG 2000 conjugate) it appears that the reaction had formed one product but that a very small amount of amine and mPEG glycidyl ether remains. This cannot be noticed with certainty in ^1H NMR spectra of the conjugates. Further, the mPEG conjugate of tetramethyl compound **110** was contaminated with inorganic salts after generation of the amines from their hydrochlorides using NaOH. Purification of this conjugate was attempted by column chromatography on silica. This also served to investigate the feasibility of chromatography of the conjugates; this proved to be successful and the pure diPEGylated conjugate was obtained in 32% yield.

3.5 Coupling to Primary Amines

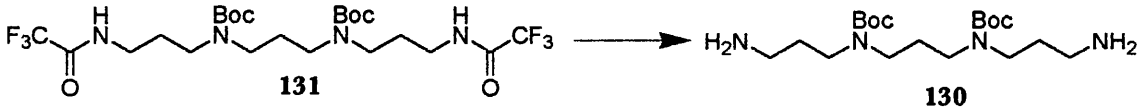
Following the successful chromatography, it seemed logical to attempt the coupling of the mPEG glycidyl ethers to thermine, protected on the middle secondary amines but bearing primary terminal amines such that, if the glycidyl ether reacts twice with one amine to any extent, this could be separated from material bearing just one mPEG chain (see scheme **33** on page 92). Whilst compounds PEGylated twice on a primary amine group may be interesting, it was thought that this would not be desirable, as DNA binding may be sterically inhibited to some extent. The DiBoc diamino compound **130** had already been prepared (scheme **42**, page 99) during the synthesis of compound **128**. This could therefore be used in the coupling reaction to see whether the mPEG glycidyl ether reacts with the primary amine once or twice. Obviously, secondary amines do react with glycidyl ethers as illustrated by the reaction with the dimethyl compound. The study of Shechter *et al* [Shechter *et al*, 1956]



130

suggests that primary and secondary amines have a similar reactivity towards epoxides, but sterically hindered amines have a significantly lower reactivity. Once the mPEG epoxide has reacted once with the primary amine, the formed secondary amine may be sufficiently hindered such that it has lower reactivity towards a further addition of mPEG. In addition, the hydroxyl β to the amine may have a deactivating effect due to the electron-withdrawing effect of the oxygen.

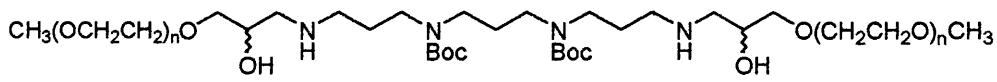
Note that compound **130** could be synthesised *via* the alternative route shown in scheme **46** by removing the TFAc groups of compound **131**, synthesised from thermine (scheme **43**).



Scheme 46 Alternative synthesis of compound **130**.

Both routes to **130** are three steps, the route from thermine has the advantage that it is quicker and near quantitative in terms of yield. However, purification posed a problem, in that it was difficult to remove all of the reaction by-product after TFAc deprotection, which is presumably trifluoroacetamide; this is discussed in more detail in chapter 7.

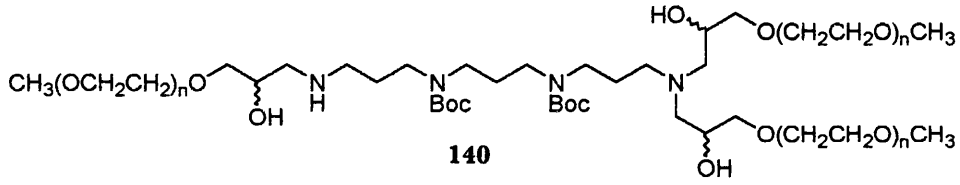
The coupling was therefore attempted using **130** and 2 equivalents of mPEG 550 glycidyl ether as before. A small-scale preliminary experiment was conducted and TLC of the reaction mixture suggested that the amines had largely reacted only once. The ^{1}H NMR spectrum was not useful in determining the extent of PEGylation, but showed the presence of a small amount of unreacted glycidyl ether suggesting that a reaction time of around 24h may not be long enough. The best evidence for the epoxide only adding once to each amine was from the FAB mass spectrum which showed the presence of an appropriate range of peaks corresponding to the correct masses for the symmetrical diPEGylated conjugate **139**, albeit at low abundance.



139 mPEG = 550

The reaction was repeated on a larger scale to allow easier chromatography and to establish whether or not the procedure was amenable to larger scale synthesis. This time two products were obtained and these were separated by column chromatography. The FAB mass spectra of both did not correspond to that obtained for the small scale preliminary experiment. One contained peaks approximately at the correct mass, but this could not be correlated to any

possible species and the abundance of the ions was very low (less than 1%). Further, the ^1H NMR spectra suggested that further PEGylation had occurred, based on the integral of the PEG CH_2 signal against the Boc CH_3 signal. Thus the two products must have been di and triPEGylated material or tri and tetraPEGylated material. Although the ^1H NMR spectra suggested tri and tetraPEGylated, mass spectra would be required to confirm this. The FAB mass spectra obtained were not useful. This is possibly because the analysis of molecules of this type and size is beyond the capabilities of the available spectrometer or because FAB is not a particularly suitable ionisation method for these compounds once the conjugate has more than two mPEG 550 chains. The samples were analysed by electrospray mass spectrometry and at first glance the spectra seemed no more conclusive than the FAB ones. However, on closer examination the peak clusters corresponding to each mass were at intervals of 22 mass units. This suggested that they corresponded to doubly charged ions, and indeed they were at the correct masses for doubly charged ions [*i.e.* ½ the apparent mass of the expected $(M+H)^+$] corresponding to tri and tetraPEGylated material, compounds **140** and **141**. It is well documented that multiply charged species often form during the electrospray ionisation process [Saf *et al*, 1997]. TetraPEGylated conjugate **141** was isolated in 15% yield and the triPEGylated conjugate **140** in 24% yield. A MALDI-TOF spectrum of **141** confirmed that it was tetraPEGylated.



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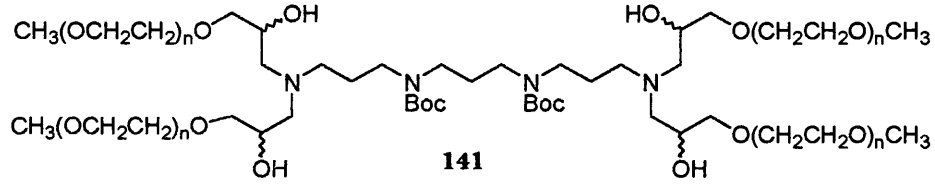


141

mPEG = 550

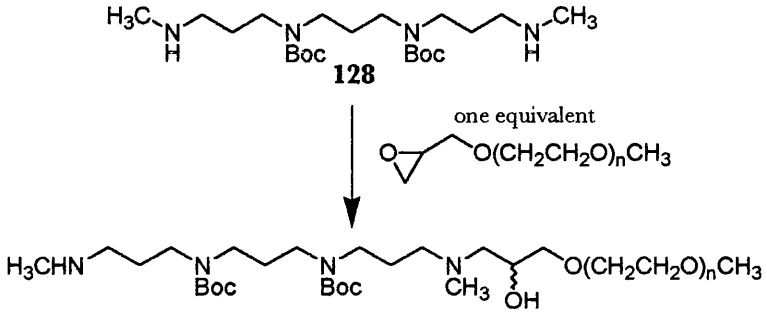
It is possible that higher PEGylation products formed in the preliminary reaction but not enough formed to be noticed by TLC; the tri and in particular the tetraPEG products stain less clearly on TLC.

4 Synthesis of Unsymmetrically Protected Thermine

A conjugate bearing a mPEG at only one end of the polyamine, an unsymmetrical conjugate, may show a stronger interaction with DNA because the polyamine may be less sterically hindered in its electrostatic interaction. Further, if the conjugation to mPEG lowers the pKa of the amine (by a deactivating effect of the hydroxy group β to the amine) such that it is less likely

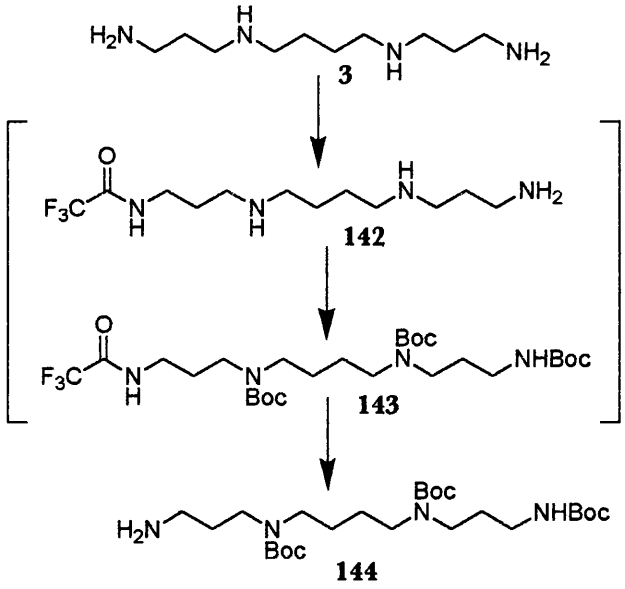
to be protonated at physiological pH, then a conjugate having just one of these amines such as an unsymmetrical conjugate may carry more positive charge at pH 7.4. Thus the synthesis of unsymmetrical conjugates is desirable.

An unsymmetrical mPEG conjugate may be synthesised using methods analogous to those used for the symmetrical diPEGylated conjugates except that an additional amine would need to be protected. Alternatively one could attempt to treat **110**, **128** or **130** with one equivalent of mPEG glycidyl ether and hope to isolate mono-PEGylated material by chromatography, for example with **128**:



Scheme 47 Possible synthesis of a monoPEGylated conjugate.

It is possible that by using this method a significant quantity of symmetrical diPEGylated material would be produced and that the yield of the desired compound would be poor. Moreover, during the course of this study a relatively simple method of producing tri-Boc spermine (scheme **48**) in one pot from spermine (50% yield) on a fairly large scale, appeared in the literature [Blagbrough and Geall, 1998].

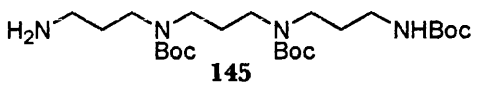


Scheme 48 Preparation of tri-Boc protected spermine**144** [Blagbrough and Geall, 1998].

The method is based on the selectivity of ethyl trifluoroacetate for primary amines over secondary amines [Xu *et al*, 1995] as the key first step in this procedure is the mono-

trifluoroacetylation of free spermine **3** by treating it with one equivalent of ethyl trifluoroacetate at −78°C then 0°C in MeOH. This affords a mixture of diTFAc and, predominantly, monoTFAc compounds. This mixture is immediately treated with four equivalents of Boc_2O which reacts with all the remaining amino groups; at this stage the mixture presumably contains a mixture of tetraBoc spermine, diBoc diTFAc spermine and triBoc monoTFAc spermine. The TFAc groups are immediately cleaved using aqueous ammonia to yield a mixture of tetra, tri and diBoc spermine. Chromatography gives the triBoc material **144** in 50% yield.

Thus triBoc thermine **145** was synthesised by an analogous procedure. Thermine was treated with one



145

equivalent of ethyl trifluoroacetate in EtOH at −78°C (dropwise over 40min) then 0°C (30min). EtOH was used here as opposed to MeOH as in the literature procedure [Blagbrough and Geall, 1998] as it was thought there was a chance it may improve the selectivity; when ethyl trifluoroacetate is used in MeOH, it presumably becomes methyl trifluoroacetate by transesterification, methyl trifluoroacetate is presumably more reactive. The mixture was then treated with Boc_2O (0°C then room temperature, overnight), then with concentrated aqueous ammonia and ammonia gas (0°C then room temperature, sealed bottle, overnight). Chromatography gave triBoc thermine in 51% yield. Consideration was given to the isolation of triBoc monotrifluoroacetyl material before the trifluoroacetamides were cleaved with aqueous ammonia as this would provide the option of methylating the amine before removing the TFAc groups. However, examination of the TLC in a number of systems at this stage showed just one spot suggesting the triBoc monoTFAc material and the tetraBoc material had the same Rf value and that separation would be difficult. Modifications to this method produced a more rapid, simpler procedure; however this did result in a moderate reduction in yield. Thermine was treated with ethyl trifluoroacetate at 0°C in THF for 15 minutes (as opposed to one hour at −78°C then one hour at 0°C, in MeOH, as in the method of Blagbrough and Geall). Then with Boc_2O (0°C then room temperature, two hours), at this stage the THF was evaporated and then the TFAc groups were removed by heating the residue in propylamine at reflux overnight. It was decided to utilise the compound for coupling to PEG at this stage *i.e.* not to methylate the primary amine as it was thought that enough monoPEG material would result from the reaction with the primary amine to be able to isolate it by chromatography and that this would provide sufficient material for this study.

5 Coupling of TriBoc Thermine to mPEG

The triBoc thermine was treated with one equivalent of mPEG 550 glycidyl ether under the same conditions employed for the synthesis of the symmetrical conjugates. The monoPEGylated

material **146** (figure **36**) was isolated by chromatography in 21% yield and the diPEGylated material **148** in 9% yield.

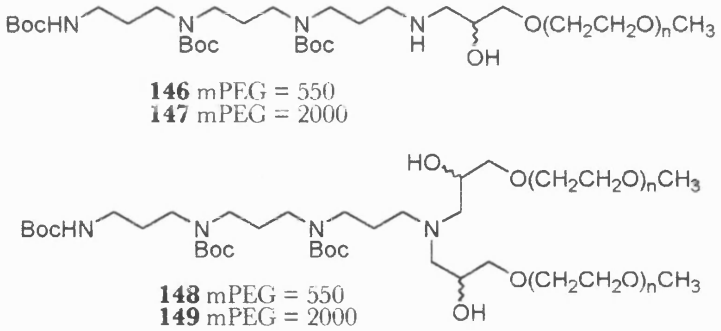


Figure 36 Unsymmetrical mPEG 550 conjugates of thermine.

FAB mass and ^1H NMR spectra confirmed the structure of these compounds. Interestingly, a higher yield was obtained when the reaction was repeated using mPEG 2000 glycidyl ether; 35% for diPEG and 28% for monoPEG. A MALDI-TOF spectrum of the diPEGylated conjugate **149** (figure **36**) confirmed its identity, this is illustrated in figure **37**. It was possible to gain a FAB spectrum of the monoPEGylated conjugate **147**.

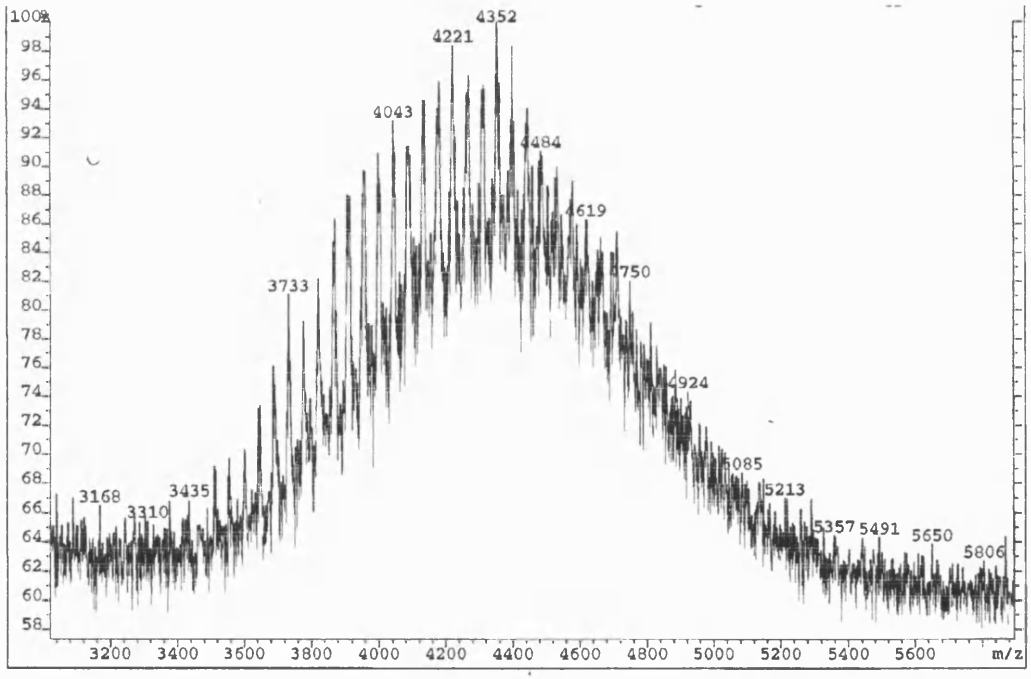


Figure 37 MALDI-TOF spectrum of conjugate **149**.

6 Deprotections

The Boc protection was easily removed from the conjugates by treatment with acid. The compounds were dissolved in dichloromethane and HCl was bubbled through for 20-30

minutes. Evaporation of the solvents gave the conjugates as their hydrochloride salts in quantitative yield. The samples were dried by lyophilisation and were semi-solids or waxes. It was immediately obvious from the ^{1}H NMR of the products whether or not the deprotection had gone to completion by the disappearance of the Boc methyl signals at δ1.45. In all cases, complete deprotection occurred within 20-30 minutes. There was no evidence from ^{1}H NMR or mass spectrometry that treatment with HCl in this way had any other effect on the molecule. All the conjugates that were deprotected are shown in figure **38**.

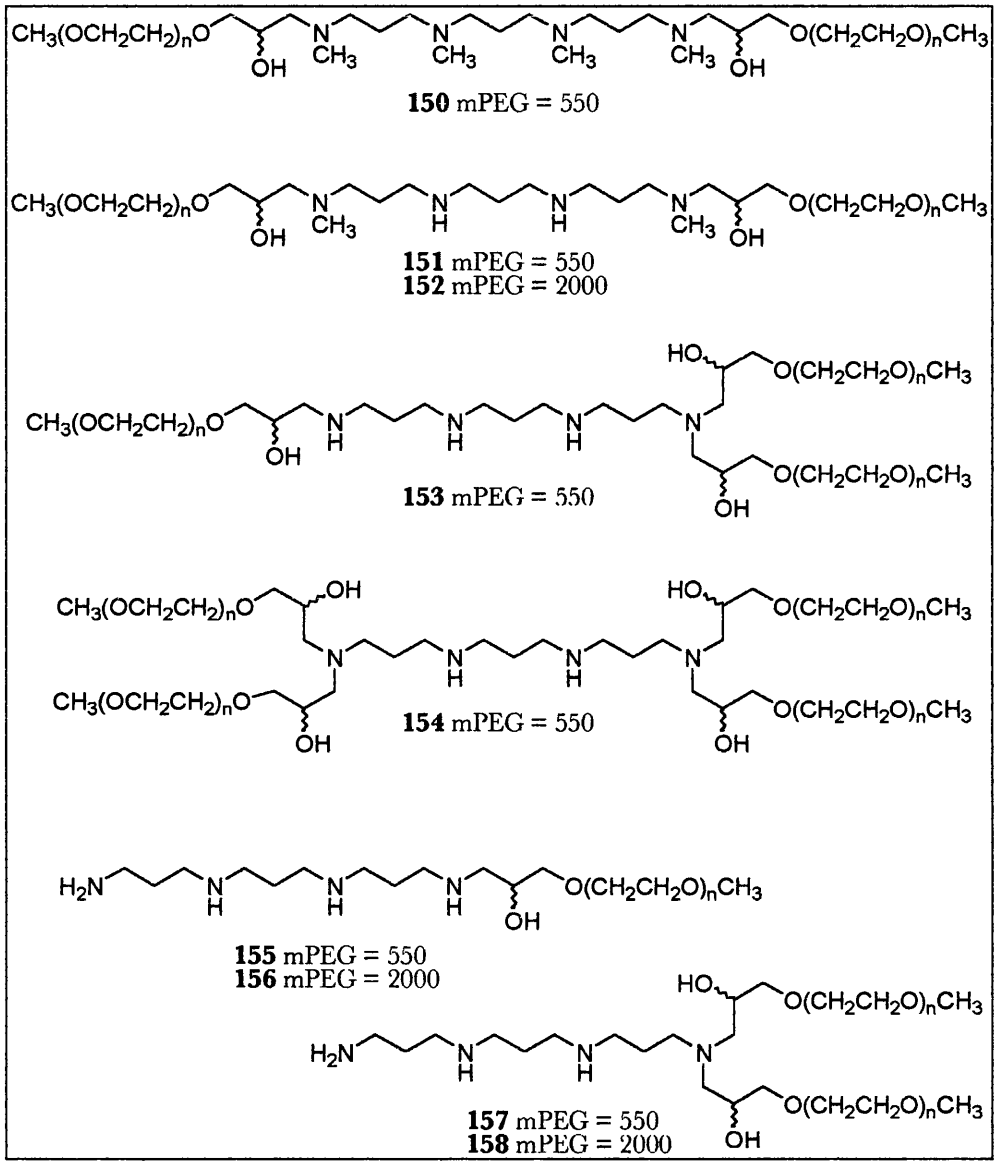


Figure 38 Deprotected mPEG-polyamine conjugates, all compounds were hydrochloride salts.

The ability of each conjugate to form complexes with DNA was examined. A spectrofluorometric assay based on the quenching of the fluorescence of an ethidium bromide-DNA complex was used. The assay principles and procedures, and the results for the conjugates described in this chapter are the subject of the next chapter.

Chapter 6:

Ethidium Bromide Assays

1 Introduction

The performance of a non-viral vector for gene delivery *in vivo* is dependant upon how the complex formed with DNA interacts with the biological milieu. Various physical and biological properties of the complex can be studied in order to gain information useful for predicting the possible fate of the complex *in vivo*. These include particle size analysis, zeta potential measurements, *in vitro* cell cytotoxicity studies, *in vitro* transfection experiments, gel electrophoretic studies and ethidium bromide exclusion/displacement assays. However, one must be careful in the interpretation of these data. Whilst physical characteristics of the complex can be correlated, to a certain extent, with transfection efficiencies *in vitro*, predictions of performance *in vivo* are considerably harder to make. Some surprising results have emerged from experiments *in vivo*, such as the activity of naked DNA in muscle [Wolff *et al*, 1990] and the high activity of complexes with the neutral polymer PVP in muscle [Mumper *et al*, 1996]. Further, there is poor correlation between *in vitro* and *in vivo* gene transfer ability. Ethidium bromide assays provide a useful, rapid measure of how strongly the vector interacts with DNA. They give us an idea of whether or not a compound is condensing the DNA; condensing agents are very efficient at excluding ethidium bromide from DNA. Thus, ethidium bromide assays were used here to study the ability of the conjugates to form complexes with DNA. This chapter describes the principles of the assay and present the results for the conjugates whose synthesis was described in chapter 5.

2 Principles of the Procedure

Ethidium bromide, figure **39**, is an intercalator. Compounds that bind to DNA, both those that are intercalators, and non-intercalating agents such as polycationic species which bind as a result of electrostatic interactions with the polyanionic DNA, can displace ethidium bromide from ethidium bromide-DNA complexes. There is correlation between the affinity of a given binding agent for DNA and the agent's efficiency at displacing ethidium bromide [Cain *et al*, 1978]. When ethidium bromide binds to DNA, there is a large increase in

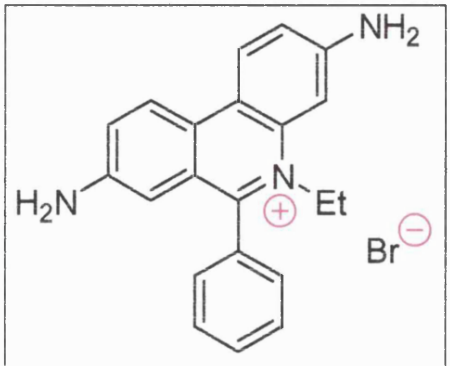


Figure 39 Ethidium bromide

the fluorescence of the ethidium bromide molecule; this fluorescence is quenched when the ethidium bromide is displaced by higher affinity compounds. Simple spectrofluorimetric methods can therefore be used to study the interaction of a range of compounds with DNA and the technique has been widely described [Cain *et al*, 1978; Gershon *et al*, 1993; Tang and Szoka,1997; Delcros *et al*, 1993; Stewart and Gray, 1992]. The mechanism by which ethidium bromide is displaced by agents which do not intercalate, such as polyamines, may be non-competitive and involve changes in DNA conformation [Delcros *et al*, 1993]. Displacement could result from polyamine binding in the major groove, minor groove or along the phosphate backbone [Stewart and Gray, 1992]; the exact mechanism is not known. The binding affinity of polycations to DNA decreases with an increase in ionic strength of the test medium as one would expect with what is primarily an electrostatic interaction.

3 DNA Condensation

In solution DNA exists as a worm-like coil. The dramatic decrease in the volume occupied by a DNA molecule provoked *in vitro* by chemical agents, particularly polyvalent cations, is referred to as *DNA condensation* [Bloomfield, 1996]. The condensed particles often assume a toroidal shape but may also form rod-like structures or globular structures. In this highly compact state the helices may be separated by just one or two layers of water. Condensation of single DNA molecules has been observed for large DNA molecules. With plasmid-size, or smaller DNA, incorporation of several molecules into the condensed structure is common and it may be difficult to distinguish condensation from aggregation or precipitation; the term condensation is usually reserved for situations where the aggregate is of finite size and orderly morphology [Bloomfield, 1996]. There are a number of agents that can cause DNA to condense, these include multivalent cations, ethanol, basic proteins (histones), cationic lipids and certain neutral polymers such as PEG and PVP. Condensation has been observed by many techniques including electron microscopy, laser light scattering, viscometry, circular dichroism and more recently by fluorescence microscopy. Agents may cause DNA condensation by a number of mechanisms; by modifying electrostatic interactions between DNA segments; by altering DNA-solvent interactions; by excluding volume to the worm-like coil; by causing localised distortion of the helix; or a combination of these effects [Bloomfield, 1996]. Polycations cause DNA condensation when 89-90% of the negative charge on the DNA is neutralised [Bloomfield, 1996]. PEG is known to induce DNA collapse [Vasilevskaya *et al*, 1995; Minagawa *et al*, 1994]. It is suggested that the contacts between DNA and PEG are considered to be thermodynamically unfavourable. Thus the solvent quality for DNA becomes poorer and there is an increase in attraction between DNA segments; at a critical concentration of PEG the abrupt contraction of the DNA occurs known as a coil to globule transition [Vasilevskaya *et al*, 1995]. In one study it was found that transition of DNA to the globule state occurred at a PEG

concentration of 250mg/ml for PEG 8000; for PEG 20000 DNA collapse was observed at *ca.* 3mg/ml and a return to the expanded coil state at *ca.* 100mg/ml [Vasilevskaya *et al*, 1995].

4 Assay Procedure

Thus an assay was used which was based on the quenching of the fluorescence of the ethidium bromide-DNA complex by the conjugates. Initially, the assay was adapted from that described by Gershon *et al* [Gershon *et al*, 1993]. In this assay, the polymer-DNA complex is formed before adding the ethidium bromide and thus measures the ability of the polymer to *exclude* ethidium bromide from intercalating DNA. This is generally described as an ethidium bromide *exclusion* assay. Briefly, complexes of calf thymus DNA and polymer are formed in buffer at pH7.4, at increasing polymer concentration and left at room temperature for 30-40 minutes. The complexes are diluted with saline (150mM) and then ethidium bromide solution is added. The fluorescence is measured immediately after vortexing the mixture. The results are expressed as fluorescence of the complex relative to a standard where no polymer is added. The results from this assay proved to be a little inconsistent within a set of data for a single conjugate. Even very small inaccuracies in the amount of DNA solution pipetted into each sample vial gave enough variation in fluorescence (with more DNA giving more fluorescence as an excess of ethidium bromide was used) to give poor data. In addition, the assay was not easily reproducible and was time-consuming. This assay may be more suited to compounds having a stronger interaction with DNA than the PEG-polyamine conjugates, such as cationic lipids. Thus an alternative method was adopted based on that described by Tang and Szoka [Tang and Szoka, 1997]. Here the compound being analysed is added to the preformed ethidium bromide-DNA complex; it is thus described as a *displacement* assay. Briefly, the ethidium bromide-DNA complex is formed in buffer in the fluorimeter cuvette equipped with a stirrer bar. The conjugate is added in small aliquots to the constantly stirring solution. A new fluorescence reading is taken 45 seconds to 1 minute after adding the polymer. The total volume of polymer solution added was no more than 2.6% of the final total volume of solution. The results are expressed as fluorescence relative to the fluorescence of the ethidium bromide-DNA complex before any polymer is added. More consistent data were produced using this method.

5 Results

5.1 Controls

Assays were run using PLL (30000-70000Da) and PVP (50000Da); these provide standards for comparison with the mPEG-polyamine conjugates. All the assays were performed in HEPES buffered saline at pH 7.4 except the assay for PVP which was performed in acetate buffer at pH 4 (the ethidium bromide assay for PVP that has been reported [Mumper *et al*, 1998] was performed at pH 4; PVP carries a small positive charge below pH 5.5). Figure **40** shows the result for PLL and figure **41** for PVP. Some of the earlier assays conducted used calf thymus DNA, however, most illustrated here were conducted using the plasmid pCMVcat (this was one of the plasmids used for the *in vivo* delivery studies). The assay for PLL was conducted using both types of DNA and the result (figure **40**) suggests that there is no difference in their behaviour in these assays.

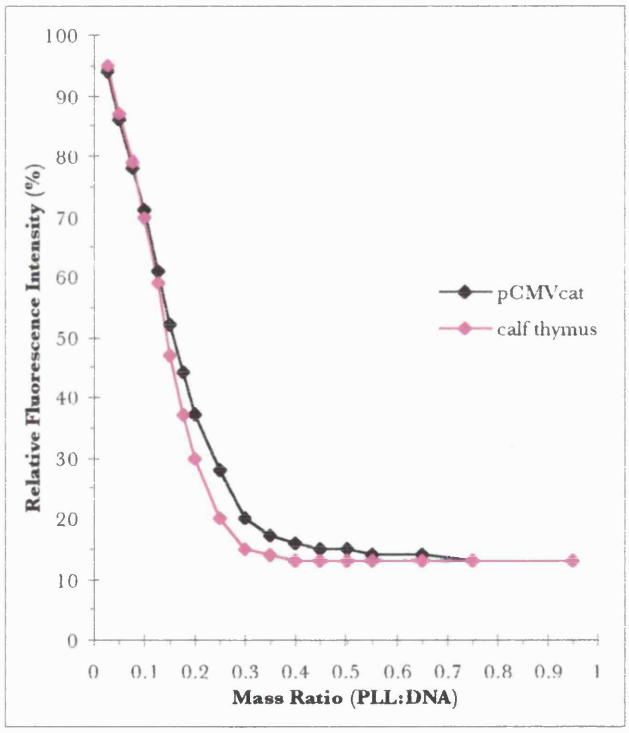


Figure 40 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of poly-L-lysine for calf thymus DNA and the plasmid pCMVcat (n = 1).

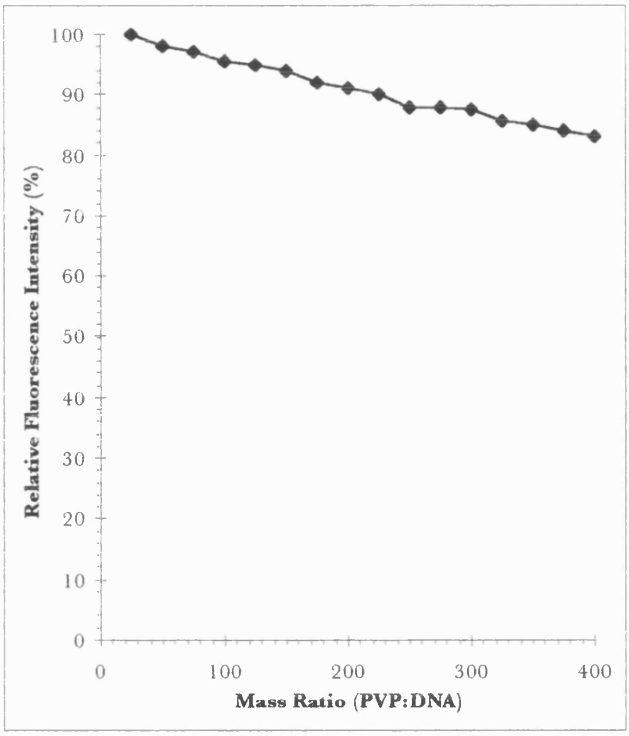
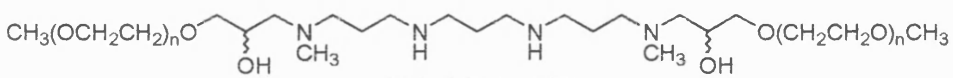


Figure 41 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of PVP 50000 (n = 1).

It can be seen that PLL and PVP differ greatly in their interaction with DNA; PLL condenses DNA efficiently and displaces much ethidium bromide at a mass ratio of *ca.* 0.2. PVP has a considerably weaker interaction and displaces little ethidium bromide at mass ratios up to 400.

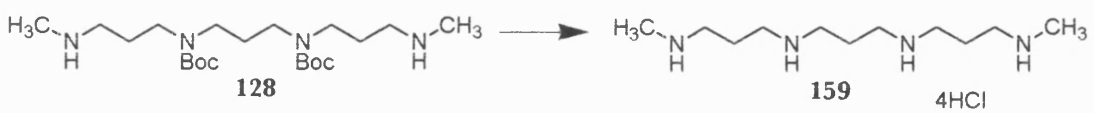
5.2 Symmetrical Conjugates

Compound **152** was found to cause no change in fluorescence when assayed.



152 mPEG = 2000

In order to establish that this lack of interaction was not due to the mPEG, other than steric effects, a sample of **128** was deprotected with HCl (scheme **49**) to yield **159** and assayed in the presence of two molar equivalents of free mPEG 2000. Figure **42** shows the exclusion of ethidium bromide from calf thymus DNA by polyamine **159** and by **159** in the presence of free PEG.



Scheme 49 Deprotection of **128**.

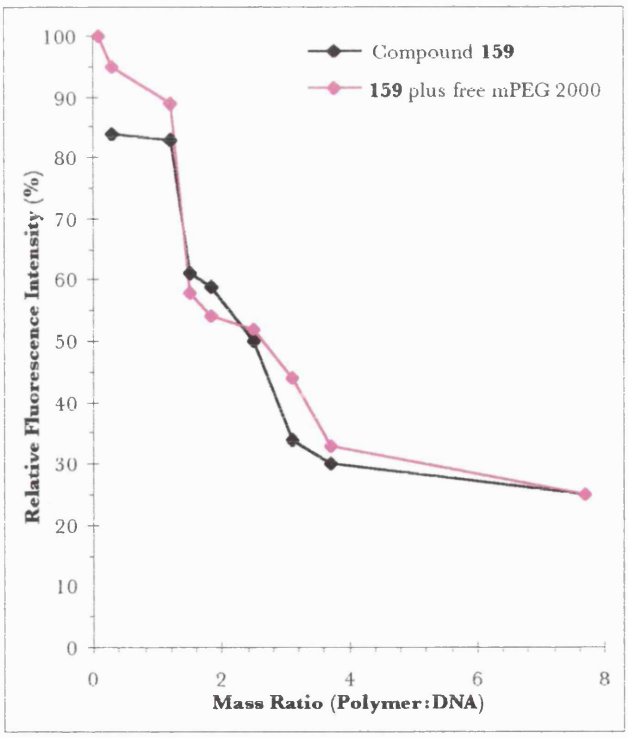
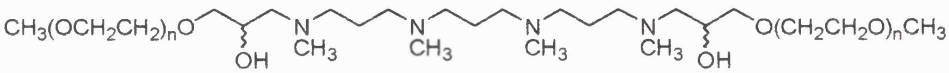
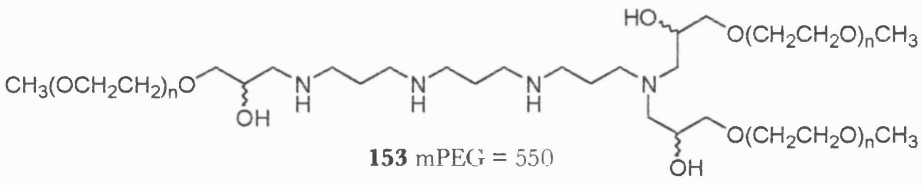


Figure 42 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of compound **159** and compound **159** plus free mPEG 2000 (n = 1).

Next, the mPEG 550 conjugates were assayed. Again there was little or no interaction observed; the results for conjugates **150** and **153** are illustrated in figure **43**.



150 mPEG = 550



153 mPEG = 550

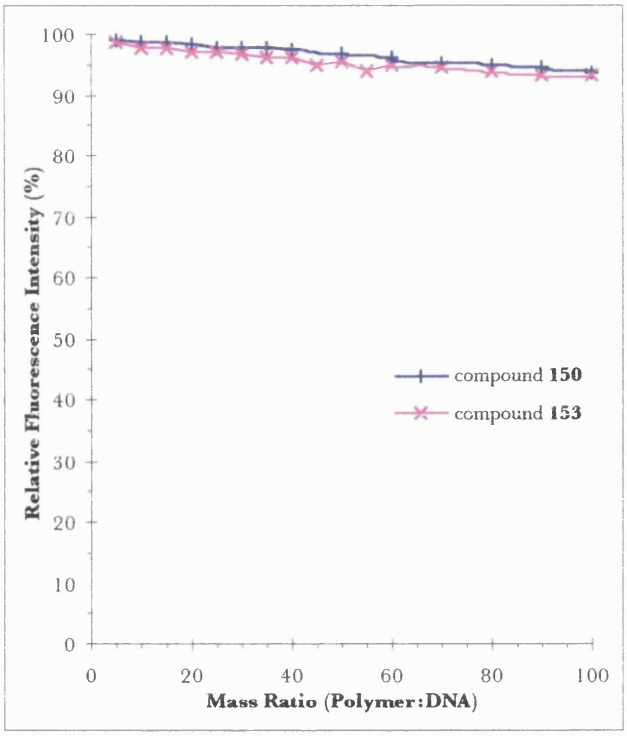
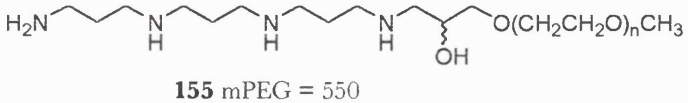


Figure 43 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of conjugates **150** (n = 1) and **153** (n = 2).

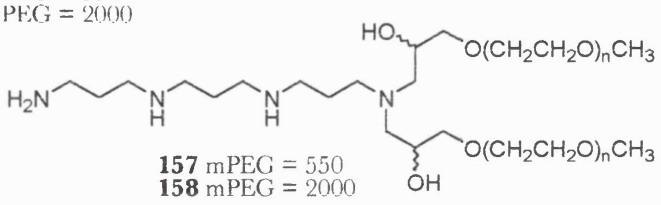
It is likely that any DNA interaction seen with these conjugates is very weak because they do not carry enough charge to induce significant conformational change in the DNA by charge neutralisation to displace ethidium bromide. In addition, it is likely that the PEG sterically hinders interaction when conjugated to both ends of a tetramine.

5.3 Unsymmetrical Conjugates

The unsymmetrically mPEGylated conjugates **155**, **156**, **157** and **158** showed a significantly greater interaction. In general the interaction became weaker with an increase in mPEG molecular weight and a higher degree of mPEGylation. Figure **44** shows the results for **155**, **156** and **157**, and figure **45** shows the results for **158**, which only showed significant interaction at high mass ratio.



155 mPEG = 550
156 mPEG = 2000



157 mPEG = 550
158 mPEG = 2000

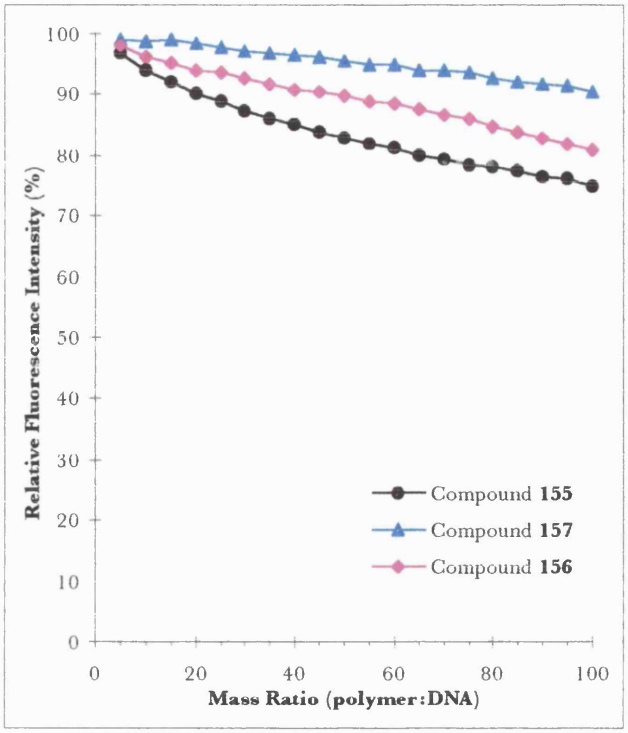


Figure 44 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of conjugates **155**, **156** and **157** (n = 3).

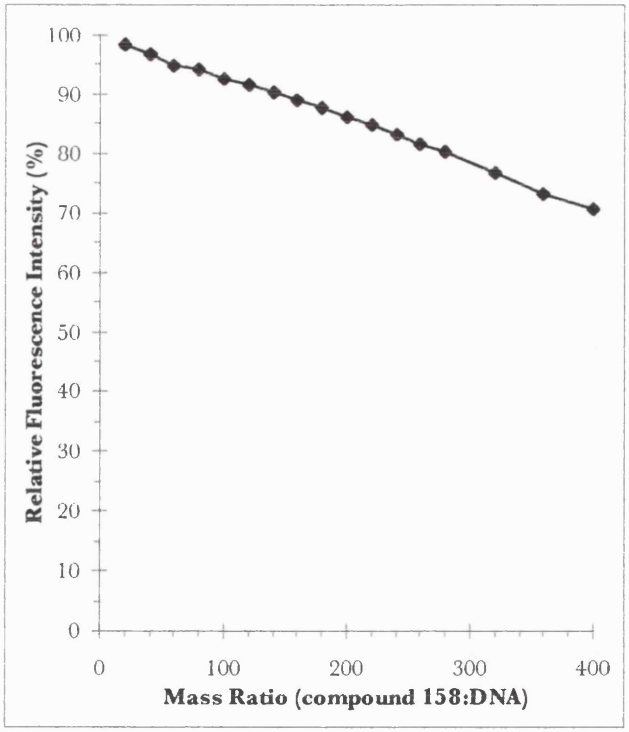


Figure 45 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of conjugate **158** (n = 2).

The pKa of each nitrogen in these conjugates is not known, thus the number of charges that each carry at pH 7.4 is not known. If the pKa value for one or more nitrogens is below 7.4 then they will not be protonated in the assay buffer solution. It was reasoned that if the assay was conducted at a lower pH and the conjugate thus carried more charge, then the DNA interaction would be stronger and more ethidium would be displaced. This proved to be the case; the assay was conducted at pH 5.5 in MES buffered saline for compound **155** and the results are illustrated in figure **46**. Thus the conjugates are not fully protonated at pH 7.4.

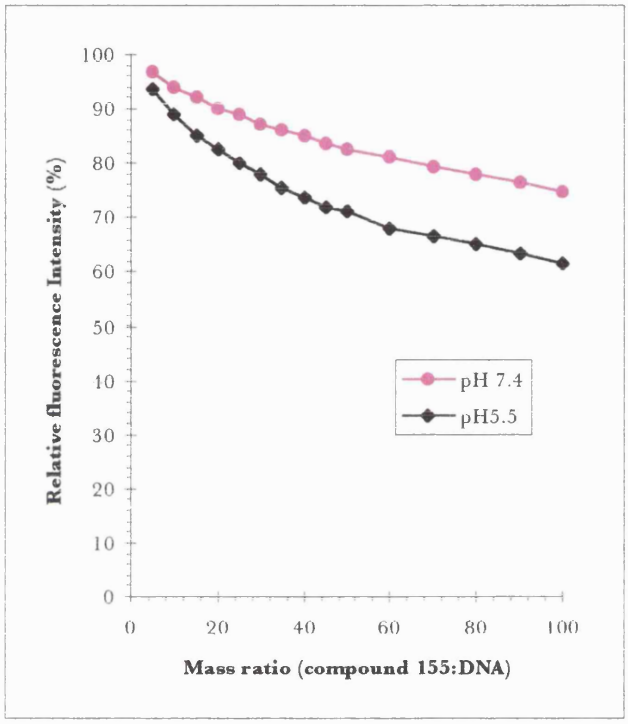


Figure 46 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of conjugate **155** at pH 7.4 and at pH 5.5 (n = 3).

Chapter 7:

Synthesis of mPEG conjugates of a Hexamine and Branched Polyamines

1 Introduction

During the course of this study, a hypothesis was put forward by Mumper and Rolland [Mumper and Rolland, 1998] which suggested that an increased binding affinity in non-condensing (PINC) systems would increase gene expression following intramuscular injection, until a point is reached, in terms of DNA interaction, when condensation of the DNA occurs leading to decreased bioavailability in muscle. They described this as an "interactive window of opportunity".

.....we propose an "interactive window of opportunity" whereby enhanced binding affinity of the future PINC systems will result in a further enhancement of gene expression after intramuscular injection due to more extensive protection of plasmids from nuclease degradation. We predict that there will be an optimal interaction beyond which either condensation of plasmids or "triplex"-type formation will occur, resulting in decreased bioavailability in muscle and, consequently, gene expression.

This is illustrated graphically in figure **47**.

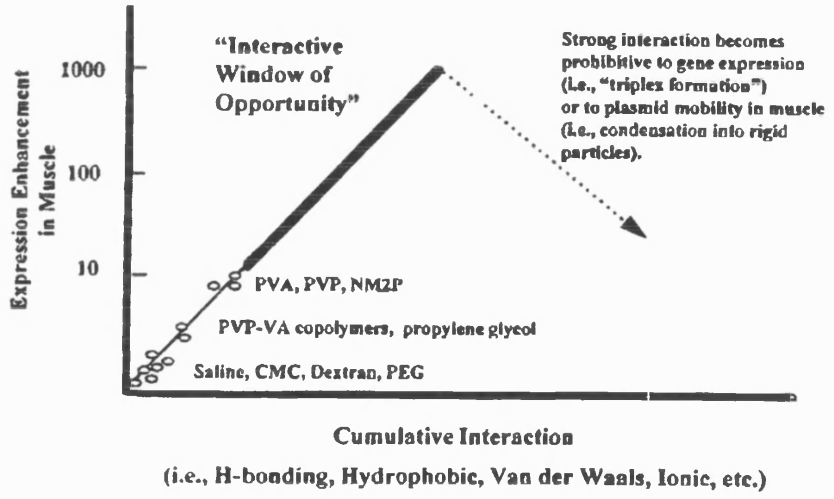


Figure 47 Future development of PINC systems: "*interactive window of opportunity*" [Mumper and Rolland, 1998]

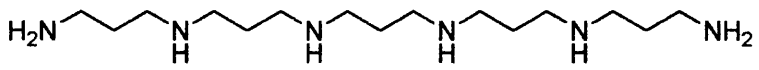
If one examines the unsymmetrical conjugates described in chapter 5 in terms of this hypothesis, it can be seen that their interaction with DNA is indeed somewhat stronger than the interaction of PVP with DNA based on the ethidium bromide assays. However, it is still considerably weaker than the cationic lipids or PLL for example. Thus it may be desirable to produce conjugate(s) having a stronger interaction with DNA than those described in chapter 5 but not as strong as the condensing, particle-forming vectors such as cationic lipids. This would provide a range of polymers having a range of affinities for DNA interaction. These polymers may have use as PINC systems for delivery of DNA to muscle, in addition they may find use in delivery to solid tumours *via* direct injection and those having a stronger interaction with DNA may be useful for delivery to other sites such as the lung, where particulate systems have shown some promise [for example: Lee *et al*, 1996].

One way to increase the interaction with DNA would be to increase the amount of positive charge carried by the conjugate. This may be achieved by increasing the number of amino groups. The interaction with DNA may be inhibited to some extent by the steric bulk of the mPEG chain, the introduction of some sort of inert spacer between the polyamine and the mPEG may circumvent this problem. Further, the use of a branched polyamine, such that the spacer and mPEG are not attached to one end of the polyamine, may also lessen steric constraints. In addition, the use of a more reactive mPEG electrophile could be used, the glycidyl ether having relatively mild reactivity.

Four conjugates were synthesised in an attempt to produce compounds with a greater affinity for DNA, two based on a linear hexamine and two branched conjugates. This chapter describes the synthesis of these compounds and their interaction with DNA.

2 Synthesis of Hexamine Conjugates

Two compounds based on the linear hexamine **160** were synthesised and coupled to mPEG in a similar manner to the tetramine conjugates described in chapter 5.

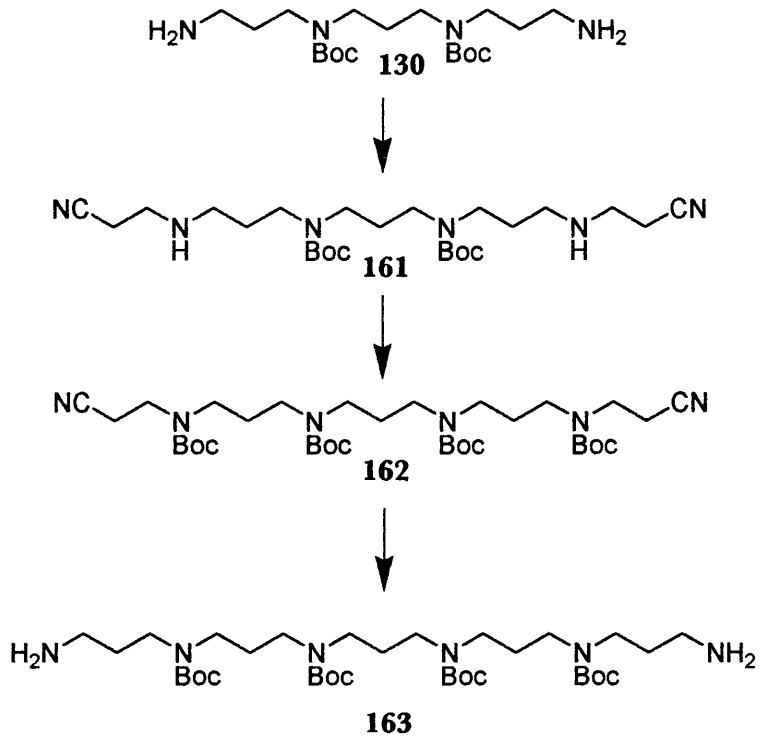


160

Obviously the coupling step would require the secondary amines to be protected. The Boc group had proved a good choice of protecting group in the tetramine routes, so again was the choice here.

2.1 Synthesis of a protected hexamine

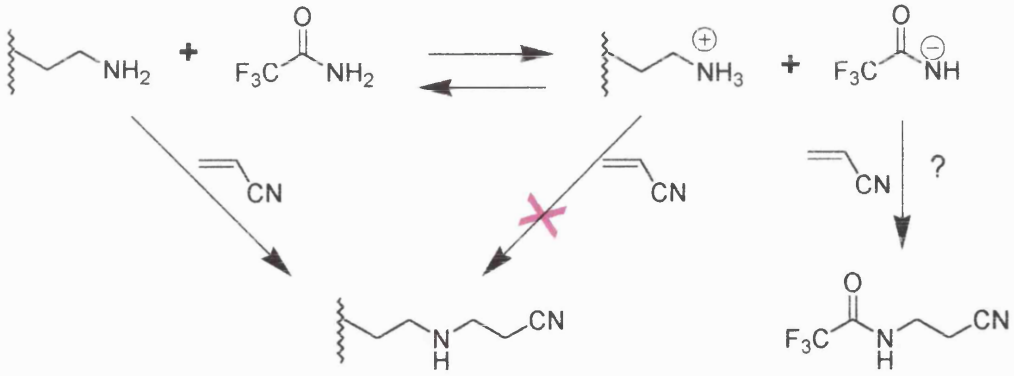
The protected hexamine intermediate **163** was synthesised by the route shown in scheme **50** from the diBoc protected compound **128** used in the synthesis of the tetramine conjugates (chapter5).



Scheme 50 Synthesis of protected hexamine **163**.

Recall that **130** could be synthesised by two routes; from propane-1,3-diamine as in scheme **42** (page 99) and from thermine as in scheme **46** (page 106). Although the route from thermine may be higher yielding, the purification of **130** produced by that route is somewhat more difficult. Removal of the TFAc groups with ammonia, presumably yields trifluoroacetamide as a by-product. It was difficult to separate this from **130** for two reasons; it has an Rf value very close to that of **130** and tended to co-elute from the silica column and it is difficult to visualise on TLC. Consequently, meticulous chromatography was required to separate **130** from trifluoroacetamide in high yield. However, it was thought that a small amount of trifluoroacetamide contamination would not interfere with the cyanoethylation reaction and that it could be more easily removed later in the reaction sequence. This proved not to be the case. Cyanoethylation in the presence of trifluoroacetamide occurred only to a small extent as judged by TLC. Therefore the nitrile reduction method (scheme **42**) was used to produce **130** for this route. This cyanoethylation reaction does not work in the presence of trifluoroacetamide, probably because trifluoroacetamide is sufficiently acidic to protonate the

amine (scheme **51**). If protonation does occur, the protonated amine will not add to acrylonitrile and the trifluoroacetamide anion may itself react with the acrylonitrile.



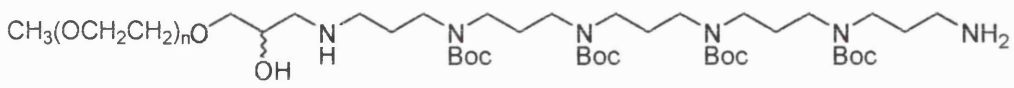
Scheme 51 Trifluoroacetamide may protonate primary amines which may not then undergo cyanoethylation.

Compound **130** was treated with acrylonitrile to give the dicyanoethylated intermediate **161**, quantitatively. The dicyano compound **161** was treated with Boc_2O in CH_2Cl_2 to give the tetraBoc protected compound **162** in quantitative yield. This was not purified at this stage but immediately reduced to the tetra-protected hexamine **163**. Dicyano compound **162** was treated with Raney nickel in methanolic ammonia at 60psi of hydrogen for 65 hours. This gave the amine **163** in 88% yield after chromatography.

2.2 Coupling to mPEG 550

Mono and symmetrical diPEGylated conjugates of hexamine **163** were synthesised. It was decided not to methylate the primary amines but rather to separate any mixtures of conjugates by chromatography after this had proved feasible with the tetramine conjugates.

MonoPEGylated conjugate

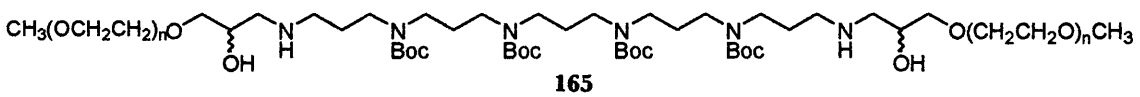


164

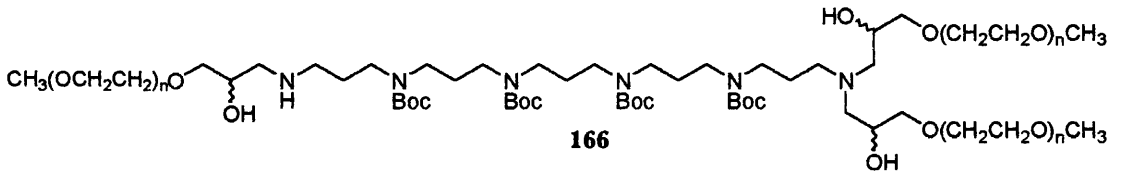
For the synthesis of the mono-PEGylated compound **164** it was decided to treat **163** with just one equivalent of mPEG 550 glycidyl ether and isolate the monoPEGylated product from the potential mixture and thus not to attempt the selective protection of one of the primary amines with Boc, followed by a mPEG coupling step. Thus the reaction was carried out in *iso*propanol at reflux for 48 hours and the monoPEGylated conjugate was isolated by column

chromatography in 32% yield† . The structure was confirmed by ^{1}H NMR and FAB mass spectrometry. Although some further PEGylated products formed, as judged by TLC, these were not recovered.

Symmetrical diPEGylated conjugate



165



166

The tetraBoc hexamine **163** was treated with two equivalents of mPEG 550 glycidyl ether. Recall that, in the equivalent reaction on tetramine, no diPEGylated material could be isolated, only tri and tetraPEGylated material. In an attempt to control the reaction, to produce some diPEGylated material, the temperature was lowered to 65°C (as opposed to 80°C in the tetramine reaction) and the reaction mixture was made more dilute. This proved to be successful as diPEGylated conjugate **165** was isolated, albeit in a modest 12% yield, together with some triPEGylated material **166**. Confirmation that the first product to elute was triPEGylated and that the second product was diPEGylated was from their mass spectra. Although it was possible to gain a useful FAB spectrum for the diPEGylated conjugate, it was not possible for **166**. However, a MALDI-TOF spectrum confirmed that it was triPEGylated. This is shown in figure **48**.

† I am grateful to Owen R. Davies, who carried out this reaction as part of a B.Pharm final year project.

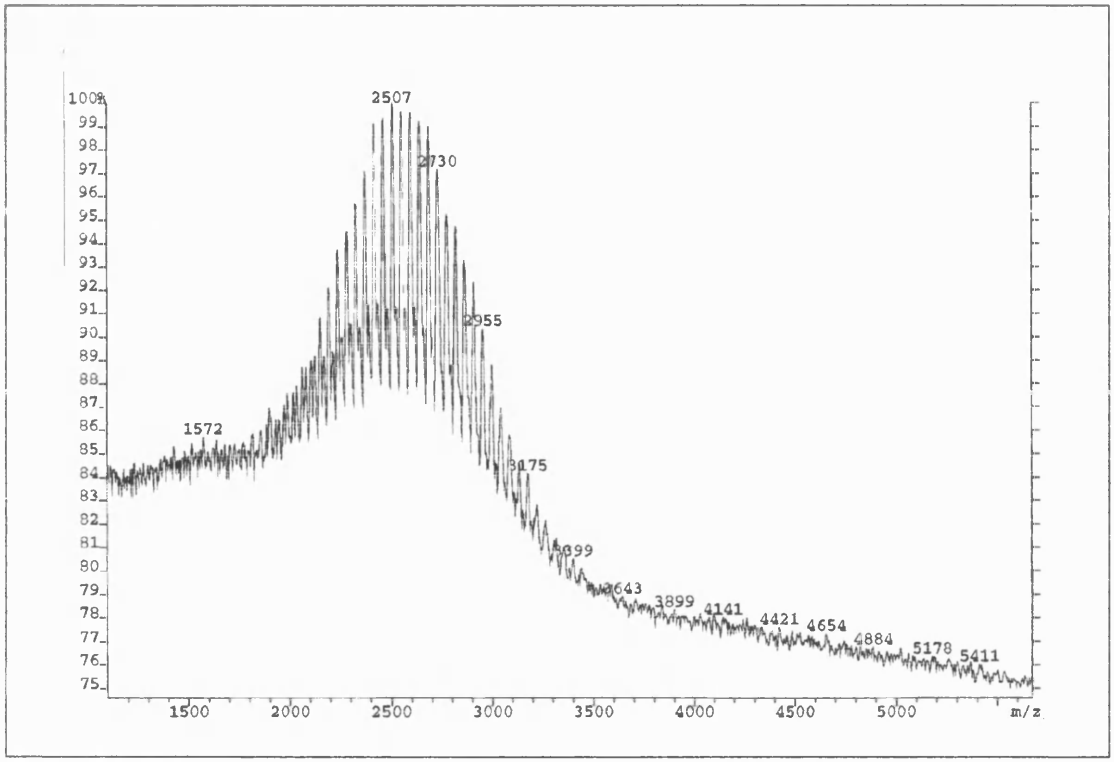


Figure 48 MALDI-TOF spectrum of conjugate **166**.

2.3 Deprotection

The conjugates were deprotected with HCl using the method described in chapter 5. A sample of hexamine **163** was also deprotected to provide a standard for use in the ethidium bromide assays. Figure **49** illustrates the deprotected compounds.

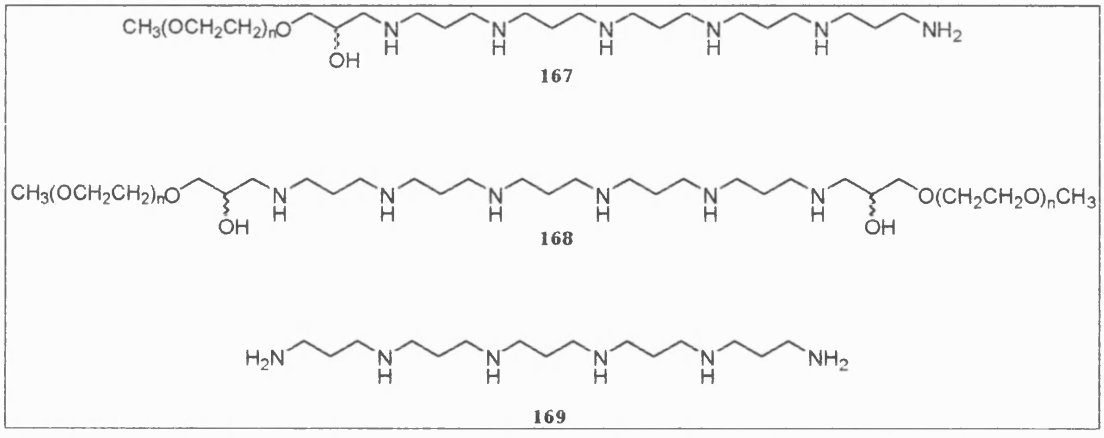
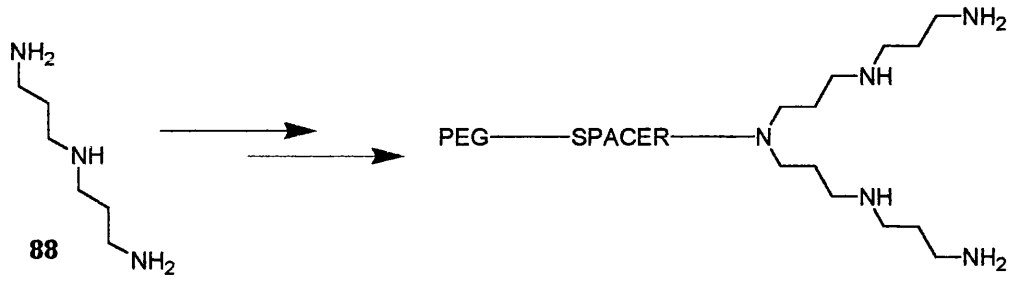


Figure 49 Deprotected hexamines, all compounds are hydrochloride salts.

3 Synthesis of Branched Conjugates

3.1 Overview

The overall strategy for the branched conjugates was to produce a pentamine bearing a spacer, to reduce the possibility of steric constraints affecting DNA interaction, to a mPEG chain on the central secondary amine (scheme **52**).



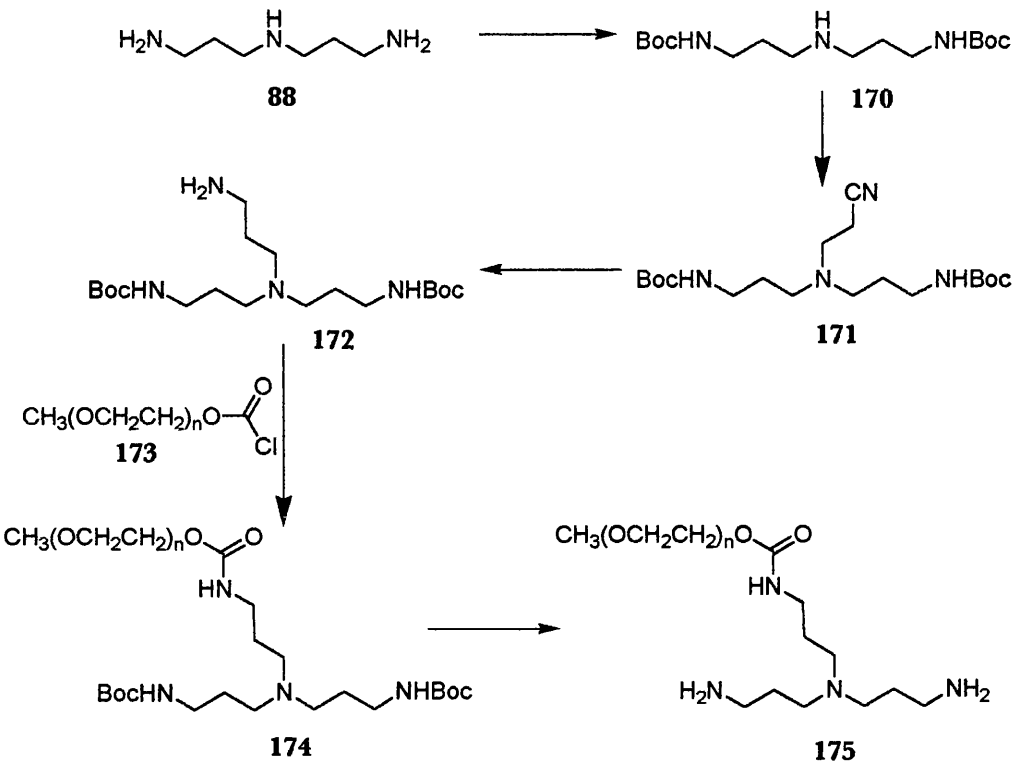
Scheme 52 Branched PEG-polyamine conjugate.

The rationale is that this could be constructed by attachment of the spacer to the secondary amine of the commercially available triamine bis(3-aminopropyl)amine **88** after selective protection of the primary amines. Following this, further aminopropyl units could be added *via* a strategy such as cyanoethylation followed by reduction or alkylation using N-(3-bromopropyl)phthalimide. Also, the branched triamine itself may have interesting properties with respect to DNA interaction, if PEGylated. An alternative strategy would be to construct the pentamine first and then attach the spacer and mPEG. Constructing the conjugates in this manner also allows for more versatility in the future design of novel vectors as the spacer may incorporate a biodegradable linker or functionality for the attachment of targeting ligands.

3.2 Synthesis of a Branched Triamine

Initial work on branched conjugates produced a PEGylated triamine with a short spacer, *via* the rapid route shown in scheme **53**.

α,ω-DiBoc bis(3-aminopropyl)amine **170** was prepared according to the method of Hu and Hesse [Hu and Hesse, 1996]. Treatment of bis(3-aminopropyl)amine **88** with 2 equivalents of 2-(*t*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON) in THF at 0°C then room temperature, gave the diBoc triamine in 54% yield after chromatography. The authors claim that the product was purified by recrystallisation. Repeated attempts to recrystallise this material failed, in that the product always contained traces of impurities. Compound **170** was cyanoethylated with acrylonitrile in THF. The reaction mixture needed to be heated to reflux, and excess acrylonitrile used, in order to obtain the cyano compound **171** in good yield (80%).



Scheme 53 Synthesis of a branched triamine.

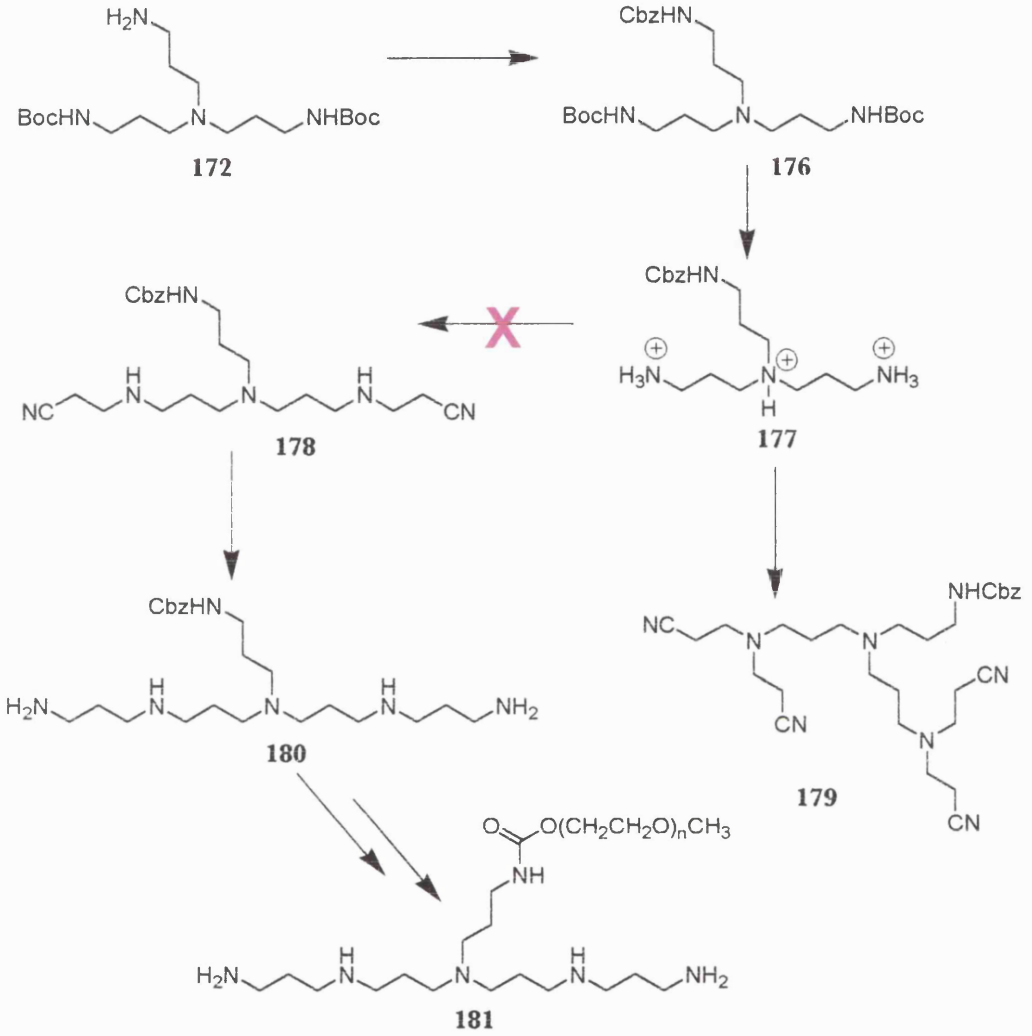
The nitrile group was reduced under 60psi of hydrogen with Raney nickel, in methanolic ammonia for 72 hours to give **172** as a blue-green oil. This was not purified, but treated with mPEG 550 chloroformate **173** to yield the PEGylated conjugate **174** in 28% yield after chromatography. The Boc protection was removed quantitatively, by dissolving the conjugate in CH_2Cl_2 and bubbling HCl through the solution to give the conjugate **175** as its hydrochloride salt.

Synthesis of mPEG 550 chloroformate

Note that the synthesis in scheme **53** used mPEG chloroformate as opposed to the glycidyl ether as in this case it was desirable that the basicity of the amine of the branch was quenched by formation of a carbamate, so that it could not take part in electrostatic interaction with DNA; also it is more reactive. Although a number of mPEG electrophiles are commercially available they are often expensive and not readily available in lower molecular weights such as 550Da. Thus the chloroformate was synthesised by treating mPEG 550 in dichloromethane with a solution of phosgene in toluene. The mPEG had been previously dried by azeotropic removal of water using toluene in a Dean and Stark trap. A large excess of phosgene was used and the product was stored in the reaction vessel; an aliquot was removed when required and the solvents and excess phosgene were evaporated immediately prior to use.

3.3 Attempted Construction of a Pentamine from Intermediate **172**

Whilst **175** is an interesting conjugate, the major target was a pentamine which would carry more charge at physiological pH. A route to a pentamine from intermediate **172** was attempted and is illustrated in scheme **54**.



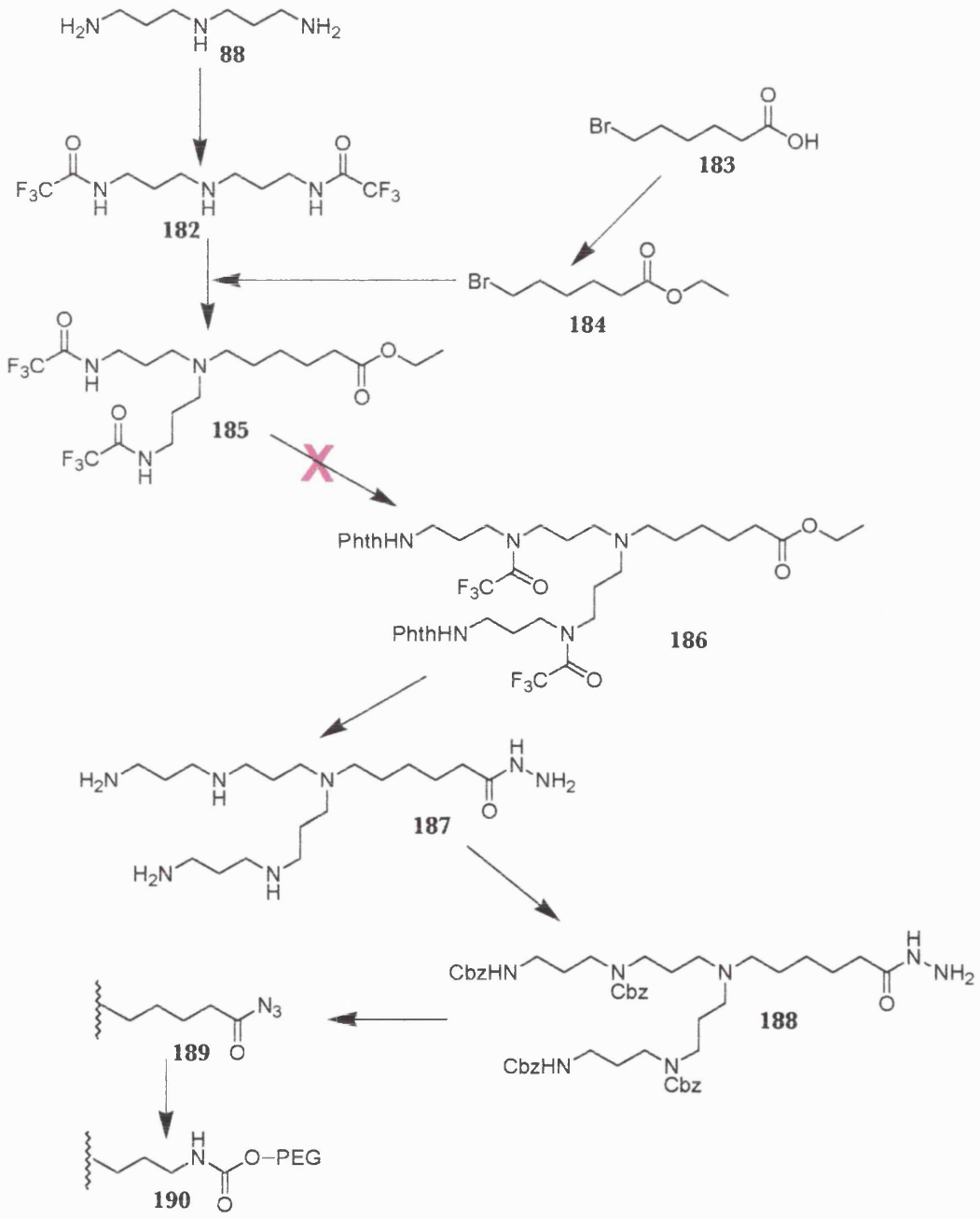
Scheme 54 Proposed synthesis of a branched pentamine from intermediate **172**.

Compound **172** was treated with benzyl chloroformate to give the Cbz protected derivative **176** in 29% yield. The low yield in the chloroformate reactions on this compound could be due to the presence of nickel contamination. Furthermore, if the nickel complex involves ammonia, then the ammonia in the reaction mixture may react with the chloroformate. Synthesis of the Cbz derivative **176** allowed removal of the Boc groups, so that building the polyamine to a pentamine could be explored; it also allowed easier purification and thus full characterisation of the intermediate. The Boc protection was removed in the usual manner with HCl to yield the triamine **177** as its hydrochloride salt. This was treated with triethylamine to generate the free base before the addition of acrylonitrile. This reaction was unsuccessful, only the tetracyanoethylated compound **179** was isolated by chromatography. This route was not

pursued further as a more efficient and versatile synthesis of a branched pentamine was progressing. Further, there is a potential problem in the route shown in scheme **54** in that the Cbz protection may not survive the reduction step of **178** to compound **180** and it is probably desirable to have a longer spacer than the aminopropyl unit.

3.4 Synthesis of a Branched Pentamine

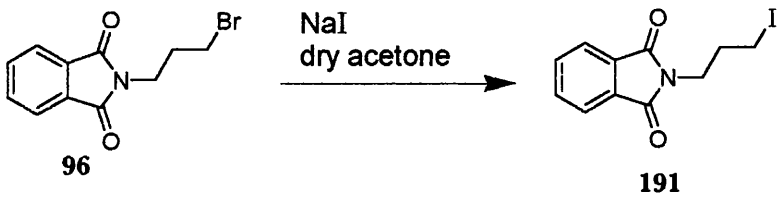
A more versatile synthesis of the desired pentamine was devised involving a strategy where the branch is attached by alkylation with a bromo compound. The first attempted route using this strategy is illustrated in scheme **55**.



Scheme 55 Proposed synthesis of a branched pentamine.

The key steps are the alkylation of the branched bis(trifluoroacetamide) **185** with N-(3-bromopropyl)phthalimide **96**, removal of all protecting groups with hydrazine and subsequent selective protection of the unmasked amines over the hydrazide in compound **187**. The hydrazide could then be converted to the azide (for example with *t*-butyl nitrite/HCl); a Curtius rearrangement followed by trapping with a mPEG would give the PEGylated conjugate **190**. This synthesis was unsuccessful as it was not possible to alkylate the bis(trifluoroacetamido) compound **185** using N-(3-bromopropyl)phthalimide **96**.

Bis(3-aminopropyl)amine **88** was treated with ethyl trifluoroacetate in EtOH to give the bis(trifluoroacetamide) **182** in quantitative yield. The ethyl ester of 6-bromohexanoic acid **184** was prepared by boiling a solution of the bromo-acid **183** in EtOH with sulfuric acid catalysis. Kugelrohr distillation gave the ester in 87% yield. The secondary amine of **182** was alkylated using this bromo-ester **184** in DMF at 80°C to yield branched compound **185**. This was an adaptation of the procedure of Muramatsu *et al* [Muramatsu *et al*, 1993] who had used ethyl 6-bromohexanoate to alkylate an N-methylamine derivative. The next step was to alkylate the trifluoroacetamide nitrogens of **185** with N-(3-bromopropyl)phthalimide as a means of building the polyamine to a pentamine. It was thought that a procedure analogous to that used to methylate compound **131** (scheme **42**, page 99) would yield the protected pentamine **186**. Thus **185** was treated with potassium *t*-butoxide in dry THF followed by N-(3-bromopropyl)phthalimide **96**, no new products could be detected in the reaction mixture. N-(3-Iodopropyl)phthalimide **191** was synthesised from the bromo compound using a Finklestein procedure (scheme **56**). It was thought that this may react more readily but again no new products formed. The fact that these halo compounds do not react with the trifluoroacetamide anions is probably largely due to the steric bulk of the alkylating species.

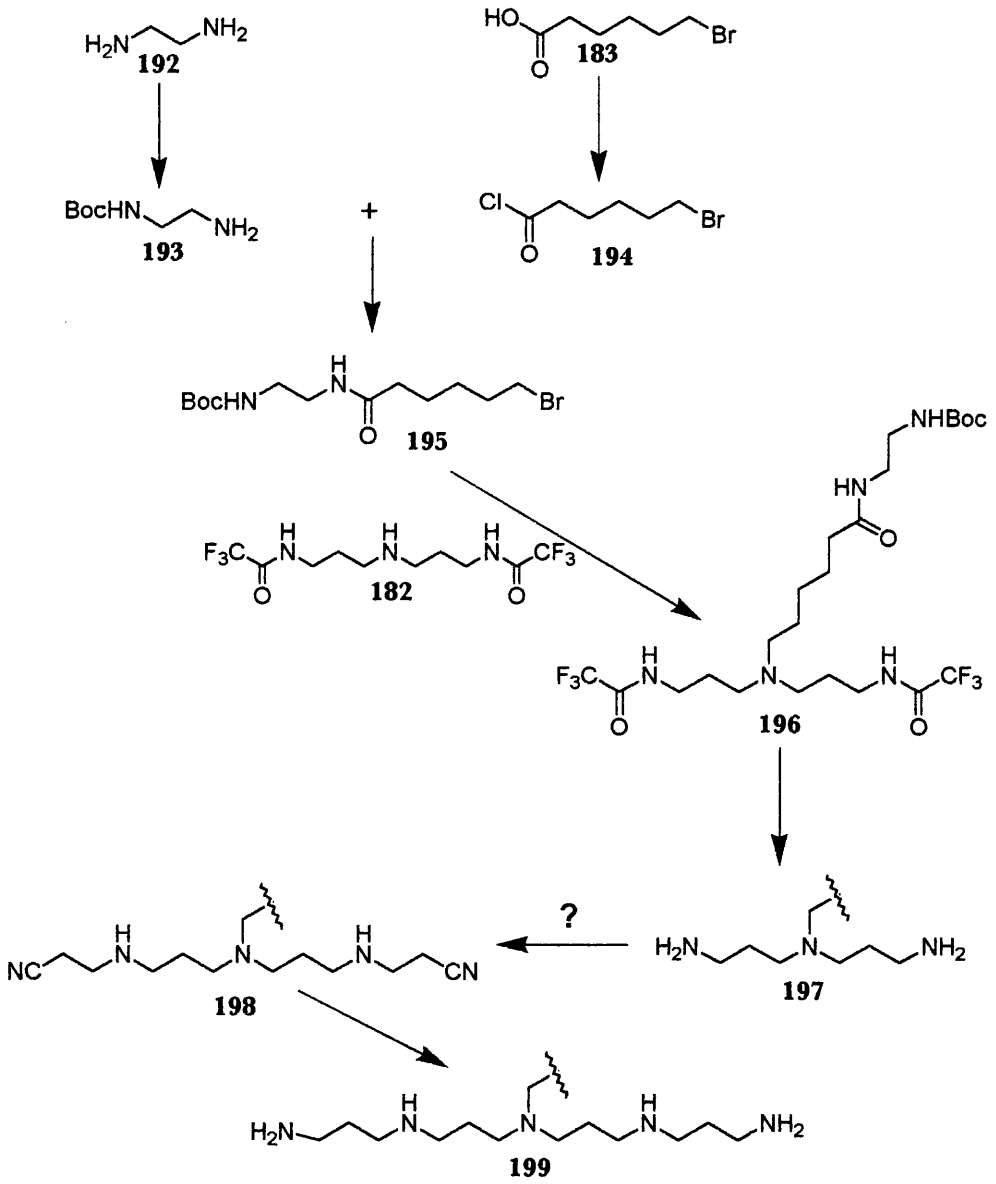


96 **191**

Scheme 56 Synthesis of N-(3-iodopropyl)phthalimide.

A new synthesis was needed and it was decided to employ the cyanoethylation and reduction sequence that had proved successful in the synthesis of linear polyamines. In addition, the use of an amine as the functional group at the end of the spacer would allow the attachment of mPEG *via* the chloroformate, the successful use of which had been demonstrated during the synthesis of the previous branched conjugate. This would require a bifunctional molecule bearing an amine at one end and an electrophilic functional group at the other. An obvious example is an amino acid such as 6-aminohexanoic acid. This could be coupled, for example, to the di-protected

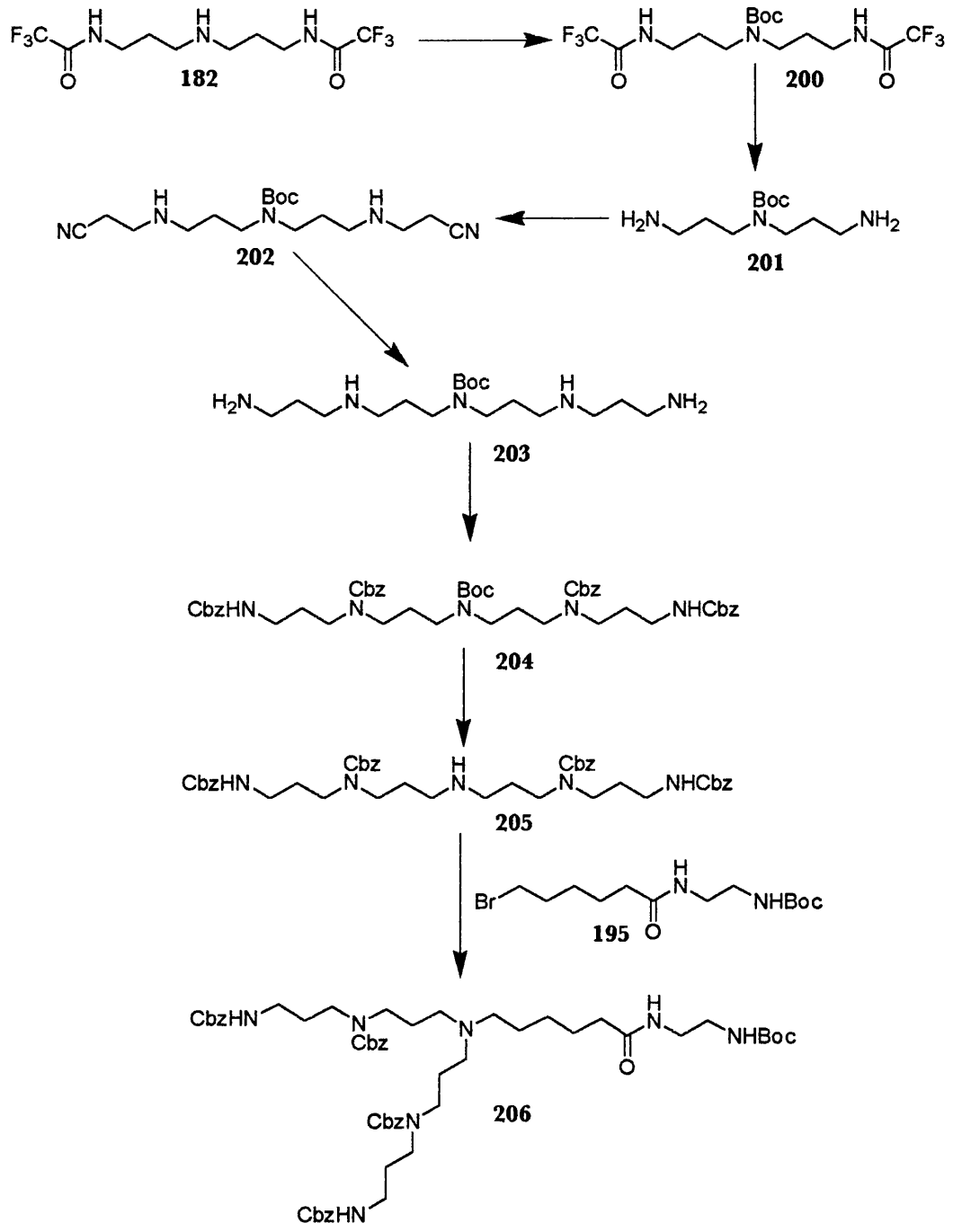
triamine **182** *via* chemistry such as a carbodiimide coupling or *via* an active ester. However, this would yield an amide and thus quench the basicity of the middle secondary amine, not allowing it the potential to be protonated and therefore take part in interacting with DNA. The amide could be reduced with borane or lithium aluminium hydride for example, but this may cause problems with protecting group selection and isolation of the products. Following the successful alkylation of **182** with the bromo-ester **184**, it was decided to use this procedure again to form the branch. The simplest way for the bromo compound to bear an amine function would be to couple the bromo-acid to a mono-protected diamine. Thus the proposed synthesis is illustrated in scheme **57**.



Scheme 57 Proposed synthesis of a branched pentamine.

t-Butyl N-(2-aminoethyl)carbamate **193** was initially prepared according to a literature procedure [Saari *et al*, 1990]. Ethane-1,2-diamine **192** was treated with $^1/_3$ equivalents of Boc_2O in THF over 30min at 0°C. Use of this procedure resulted in a significant quantity of diBoc protected material. Although it was found that this could be crystallised from the mixture, a more satisfactory procedure was to utilise a *ca.* 20-fold excess of ethane-1,2-diamine **192** in the reaction. This ensured that no diBoc material formed; the excess diamine was easily removed by extraction into water. Some of the desired monoBoc material is probably lost during water extraction but, given the simplicity of the synthesis and the low cost of the starting materials, this is acceptable. The next step was to couple this to 6-bromohexanoic acid **183** forming the amide **195**. There is much literature on amide bond formation owing to the vast amount of work that has been done in the area of peptide synthesis. One of the simplest ways to form an amide bond is by using the acid chloride. This is little used in peptide chemistry, largely owing to the potential for racemisation and side reactions such as N-carboxy anhydride formation. This should not be a problem here, thus the acid chloride **194** was the active species of choice. The acid chloride was generated using oxalyl chloride with DMF catalysis. It was not isolated but was allowed to react with the monoBoc ethane-1,2-diamine *in situ*. The amide was conveniently recrystallised from the reaction mixture, after aqueous work up, in 68% yield. The bromo-amide **195** was used to alkylate the diTFAc protected triamine **182**. The triamine was treated with **195** in DMF at 80°C for 18 hours, this gave the branched polyamine **196** in 68% yield after chromatography. The next two steps were to be removal of the TFAc groups then cyanoethylation of the free amines. However, in a preliminary experiment to remove the TFAc groups with ammonia/MeOH, it was not possible to move the deprotected product on TLC with any solvent useful for column chromatography and therefore it was not possible to purify it conveniently (although ^{1}H NMR of the crude material suggested that the deprotection was successful, as did the FAB mass spectrum). Given that the next step was to be cyanoethylation and given that such a reaction had previously proved to be difficult in the presence of trifluoroacetamide, it was considered at this stage that this route would not provide an adequate synthesis of the desired compound. Moreover, it is possible that the other amide in the molecule may suffer hydrolysis to some extent in the TFAc deprotection step, and/or this amide may also be affected in the reduction step which uses methanolic ammonia.

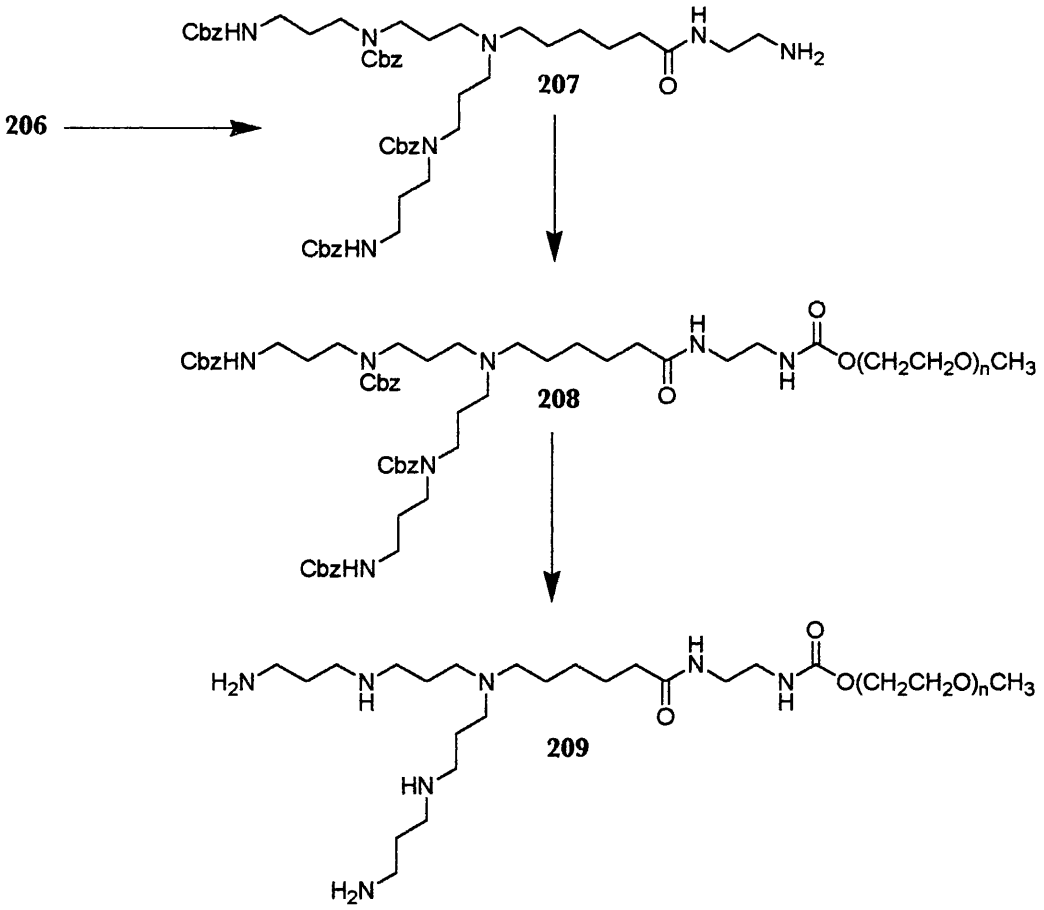
Given these difficulties it was decided to use an approach where the pentamine is constructed first and then the branch is alkylated on before coupling to mPEG. The next proposed route to the fully protected pentamine is illustrated in scheme **58**.



Scheme 58 Synthesis of fully protected branched pentamine **206**.

Bis(trifluoroacetamido) triamine **182** was treated with Boc_2O in dichloromethane to yield **200** quantitatively. The TFAc groups were removed with aqueous ammonia in MeOH and the resulting diamine was cyanoethylated. As predicted, the cyanoethylation did not proceed to completion as it was not possible to remove all the trifluoroacetamide by chromatography. In order to overcome this problem, it would have been possible to use alternative protecting groups such as Cbz but this would involve more demanding syntheses. The materials required for this synthesis are cheap and readily available and it considered easier to proceed with the planned synthesis on a larger scale and accept the low yield and need to chromatograph the

cyanoethylated intermediate **202**. Furthermore, it was found that it was possible to proceed from **182** to **202** in essentially a one-pot procedure, without isolation of the intermediates in 30% yield and moreover, that the problem of cyanoethylation in the presence of trifluoroacetamide could be ameliorated by the addition of potassium carbonate to the reaction mixture. The dicyano compound **202** was reduced with Raney nickel in methanolic ammonia. The resulting pentamine **203** was not isolated but immediately converted to compound **204** by treatment with dibenzyl dicarbonate in 60% yield after chromatography. The Boc protection was removed in 91% yield using HCl. The unmasked secondary amine was then alkylated with the protected bromo amide **195** in DMF at 80°C, as before, to give the fully protected branched polyamine **206** in 67% yield. The Boc protection was removed in 87% yield using HCl to give amine **207** (scheme **59**). The free amine was allowed to react with excess mPEG 550 chloroformate, synthesised as described before, giving the PEGylated conjugate **208** in 42% yield after chromatography. The Cbz protection was removed quantitatively by treatment with hydrogen at 60psi over Pearlman's catalyst to yield **209** and the conjugate was converted to its hydrochloride salt with HCl; thus the synthesis of a branched pentamine-mPEG conjugate was achieved.

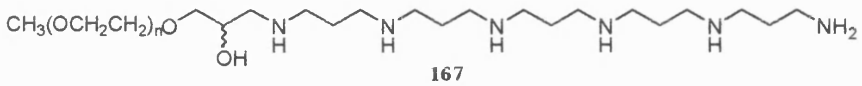


Scheme 59 Conjugation and deprotection of branched pentamine **206**.

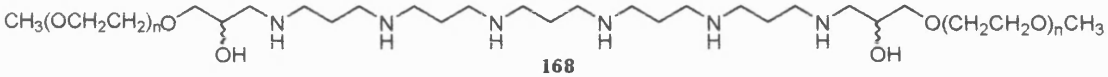
4 Ethidium Bromide Assays

The conjugates described in this chapter were assayed for their DNA binding using the ethidium bromide displacement assay described in chapter 6.

4.1 Hexamine Conjugates



167



168

169

Figure **50** illustrates the result for the monoPEGylated conjugate **167**, figure **51** for diPEGylated conjugate **168** and figure **52** for the free hexamine **169**; these results clearly show the effect that PEGylation has on the binding of polyamines to DNA.

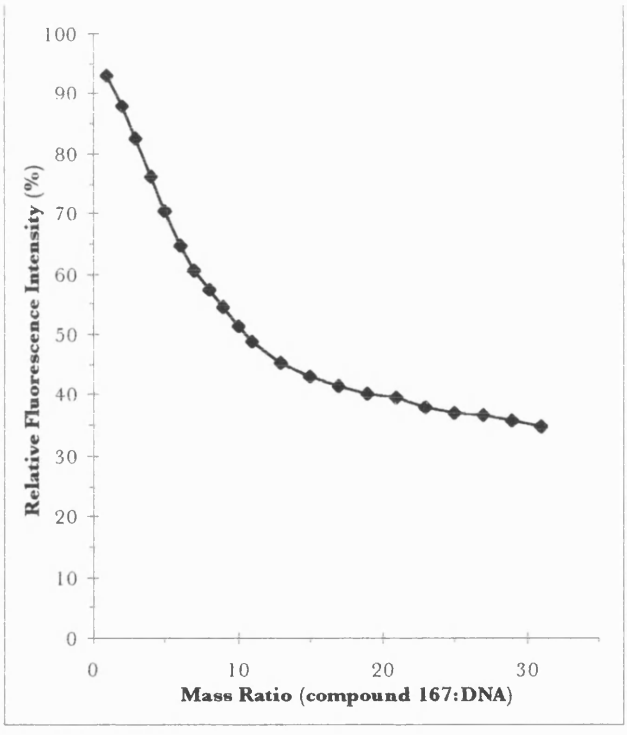


Figure 50 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of compound **167** (n = 3).

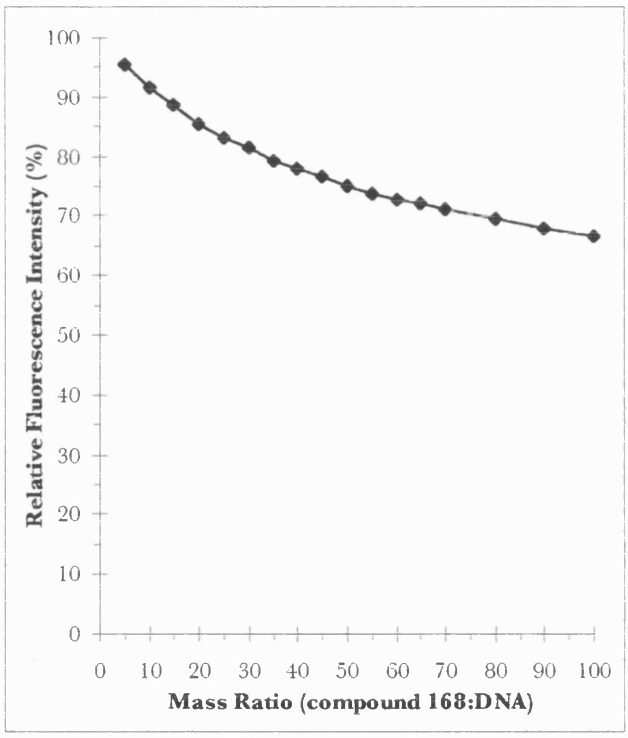


Figure 51 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of compound **168** (n = 3).

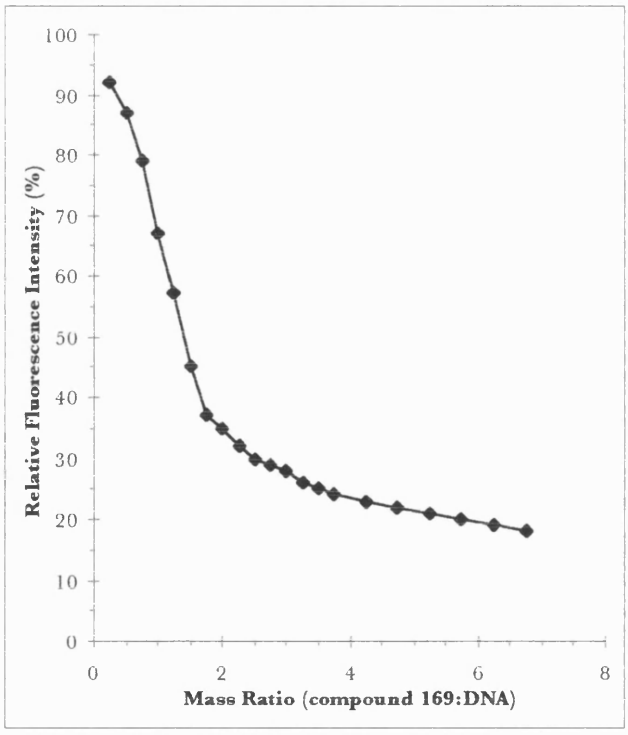


Figure 52 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of compound **169** (n = 1).

4.2 Branched Conjugates

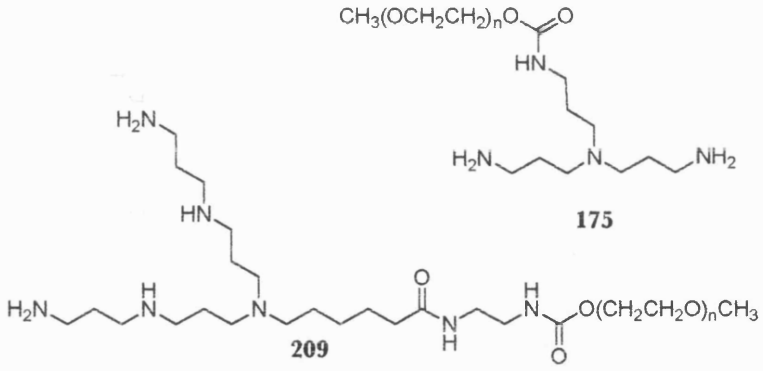


Figure **53** shows the ethidium bromide exclusion results for the branched conjugates. DiPEGylated hexamine **168** is included for comparison. As would be expected, the branched triamine **175** has a much weaker interaction with DNA than the branched pentamine **209** as it carries less positive charge. The slightly weaker interaction of the hexamine conjugate **168** compared to the branched pentamine **209** is explained by steric effects and the fact that it probably has less charge per unit mass (as it possesses two mPEG chains as opposed to one for **209**).

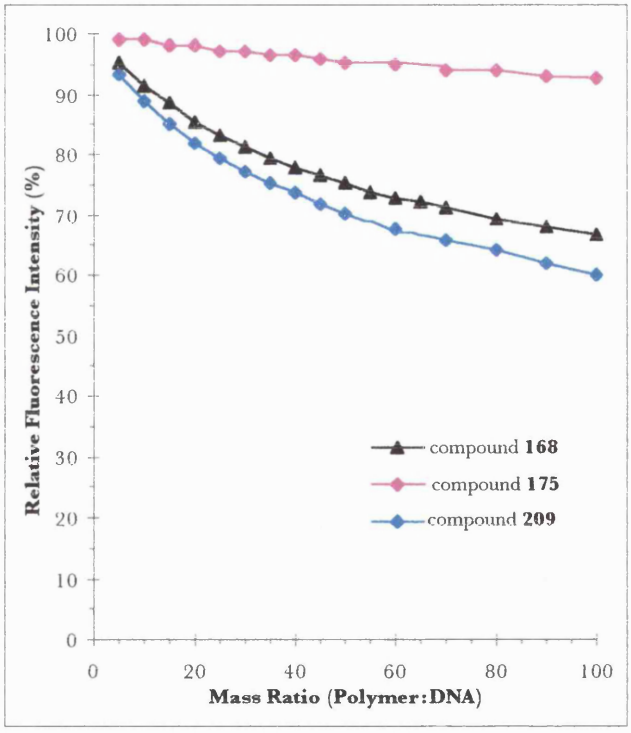


Figure 53 Changes in the fluorescence intensity of an ethidium bromide-DNA complex in the presence of increasing amounts of compounds **168** (n = 3), **175** (n = 2) and **209** (n = 3).

Chapter 8 gives further discussion on the significance of the results of these assays and those shown in chapter 6.

Chapter 8:

Gene Delivery Studies *in vivo* and General Conclusions

1 Introduction

This chapter gives a brief description of the *in vivo* gene delivery studies that were carried out using the conjugates whose synthesis and DNA interaction has been described. The results of these studies are presented along with conclusions drawn from the synthesis, the ethidium bromide assays and the *in vivo* work.

Some of the conjugates were tested for their ability to facilitate the introduction of β-galactosidase and chloramphenicol acetyl transferase reporter genes into the hind leg muscle, and into RIF-1 tumours of mice. The conjugates were compared to PVP and naked DNA.

2 Method for *In Vivo* Studies

I am grateful to Mr David A. Milroy and Dr Pauline J. Wood for performing these experiments.

Implantation of RIF-1 tumour cells

Female C3H mice were obtained from Charles River at 6-7 weeks of age and allowed to acclimatise for one week prior to the experiments. Murine fibrosarcoma RIF-1 cells were cultured in RPMI 1640 medium supplemented with 15% foetal calf serum, penicillin and streptomycin. The cells were not passaged more than 7 times prior to trypsinisation for implantation. Prior to implantation, the mice were lightly anaesthetised using intraperitoneal Hypnorm. A suspension of RIF-1 cells (containing approximately $2x10^5$ RIF-1 cells in 0.05ml of phosphate buffered saline) was injected intradermally midway along the shaved back of the mouse, approximately 2cm from the base of the tail. To obtain an acceptable tumour implant, the cell suspension should leave a spherical bump devoid of any tracks caused by leakage upon needle removal. The tumours are visible after approximately seven days, with experimental size being reached 12-14 days post-implantation. One day before tumour injection, the mice were weighed and the dimensions of the tumour recorded. The average tumour was approximately 6-7mm in diameter and 250mg in weight at this stage. The mice were then randomly assigned to the different treatment cages.

Injection of the DNA formulations

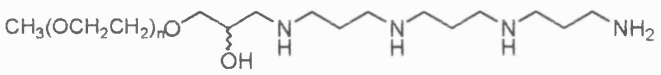
Injections were made using a sterile Hamilton syringe fitted with a 31G1/2 needle and a calibrated dosing clamp which ensured that each injection was 20µl. Each 20µl injection contained 50µg of DNA (pCMVβ-gal or pCMVcat) alone or in combination with a polymer adjuvant adjusted to be isotonic. Five days before the tumours were dosed, an injection of the formulation was made into the right hind leg muscle of each mouse. To facilitate muscle injection the mice were anaesthetised using a combination of Hypnovel and Hypnorm intraperitoneally and the right hind thigh was shaved. The needle was left in place for a short period to prevent formulation being expelled by the tissue. The tumours were injected five days after the muscle, using the same procedure, but without anaesthetising the mice. Tissue harvesting following culling (by cervical dislocation) of the mice was performed two days after the tumours were injected and thus seven days after the muscle was injected. Eight mice were used for each formulation. The negative control represents the injection of the expression construct from which the reporter gene has been removed. The positive control is the injection of DNA with no polymer adjuvant.

Tissue processing for gene expression

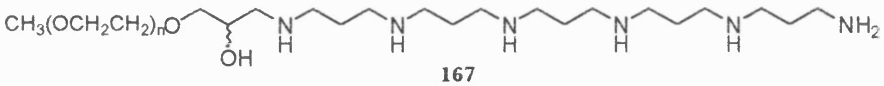
Tumour and muscle tissue was processed for gene expression using one of two methods depending on whether the tissue was dosed with a plasmid encoding for the *cat* reporter gene or the *β-Gal* reporter gene. Both reporter gene assays share a common tissue preparation protocol. Following weighing, the tissue is homogenised in lysis buffer (0.1M potassium phosphate buffer pH 7.8, 0.2% Triton X-100). The tissue suspension is then centrifuged at 4°C to remove the cellular debris. The protein in this supernatant is analysed using the Bio-Rad D_c Protein Assay kit. The supernatant is then assayed for either β-Gal or CAT depending on the plasmid injected. The β-Gal assay was carried out using the Tropix (Bedford, MA) Galacto-Light Plus chemiluminescent assay which yields Relative Light Units (RLU). While CAT activity was assayed for using a CAT ELISA colorimetric enzyme immunoassay from Bochringer-Mannheim (Mannheim, Germany). For both reporter genes, expression was normalised to either total muscle protein, tissue weight or tissue volume.

3 Results of *In Vivo* Studies

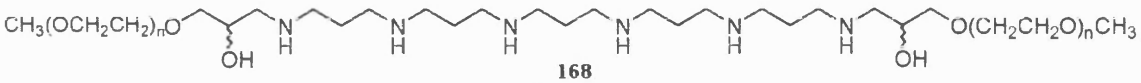
Constraints on time and resources allowed the *in vivo* testing of only three of the conjugates. These were compounds **155**, **167** and **168**. The data for these studies are presented as box plots; on the lines that connect the data for each formulation, one star (*) indicates a 95% probability of significance and two stars (* *) indicate a 99% probability of significance.



155



167



168

Figure **54** shows the result for compound **155** in muscle for transfer of pCMVβ-Gal. In this experiment neither PVP nor **155** show significant enhancement of gene expression over naked DNA.

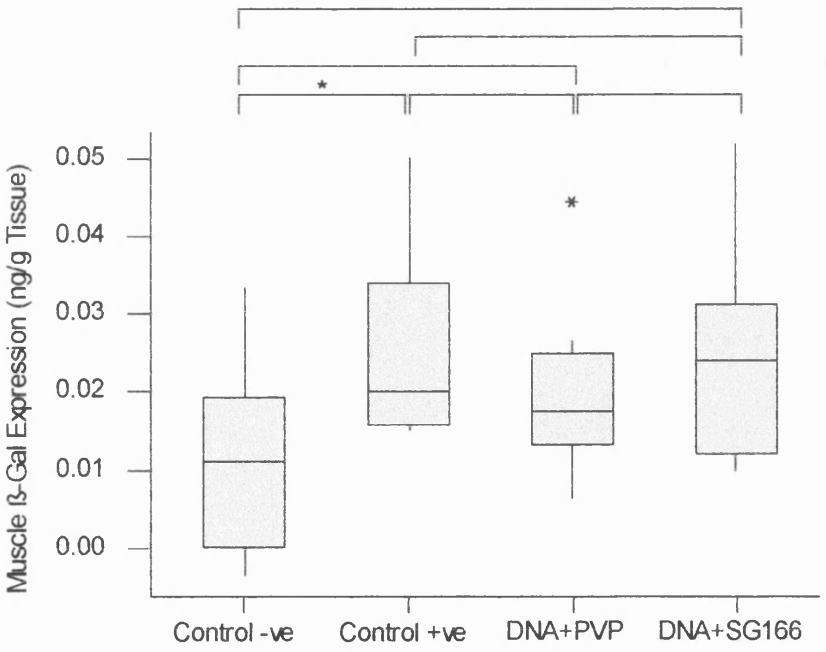


Figure 54 β-galactosidase expression in mouse muscle after direct injection of various formulations of pCMVβ-Gal. In this graph compound **155** is denoted SG166.

Figure **55** shows the result for pCMVβ-Gal transfer into tumour using compound **155** compared to PVP and positive and negative controls. Compound **155** produced a significant enhancement of gene expression over naked DNA. Although enhancement of expression compared to PVP is also seen, it is not significant when subjected to statistical analysis.

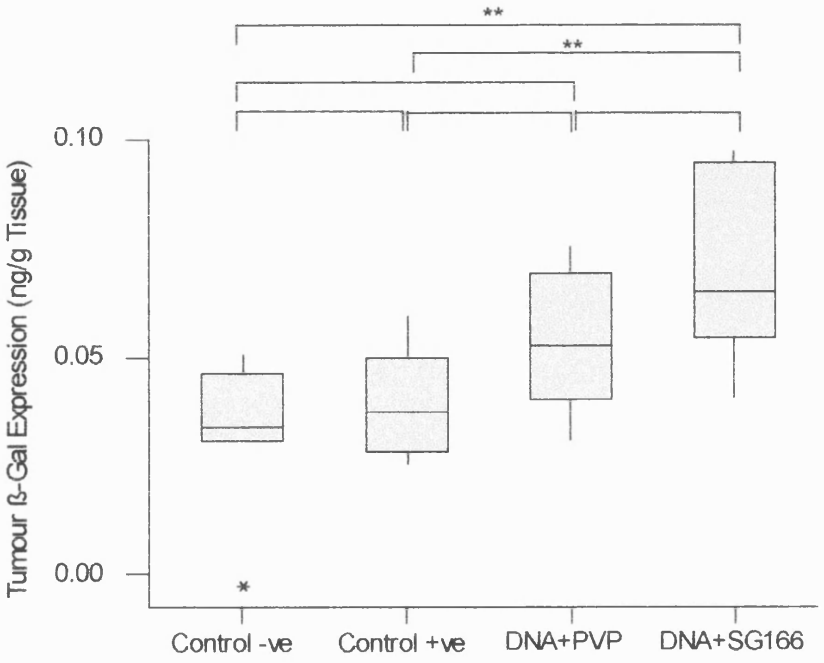


Figure 55 β-galactosidase expression in RIF-1 tumours after direct injection of various formulations of pCMVβ-Gal. In this graph compound **155** is denoted SG166.

Figures **56** and **57** show experiments where compounds **155** and **167** are compared to naked DNA and PVP in tumour (figure **56**) and in muscle (figure **57**), this time using the pCMVcat reporter plasmid. Compounds **155** and **167** both significantly enhance expression in tumour, whereas no activity is seen in muscle.

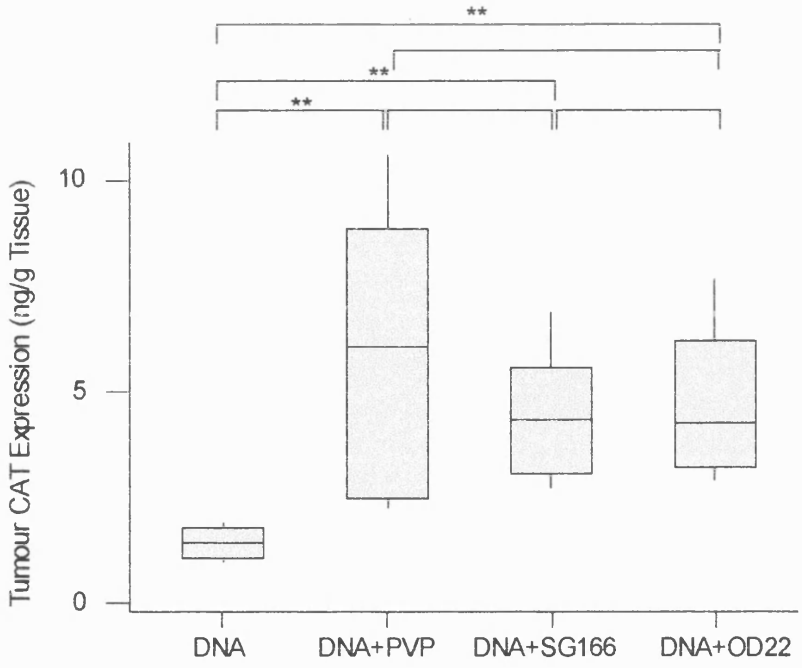


Figure 56 Chloramphenicol acetyl transferase expression in RIF-1 tumours after direct injection of various formulations of pCMVcat. In this graph compound **155** is denoted SG166 and compound **167** is denoted OD22.

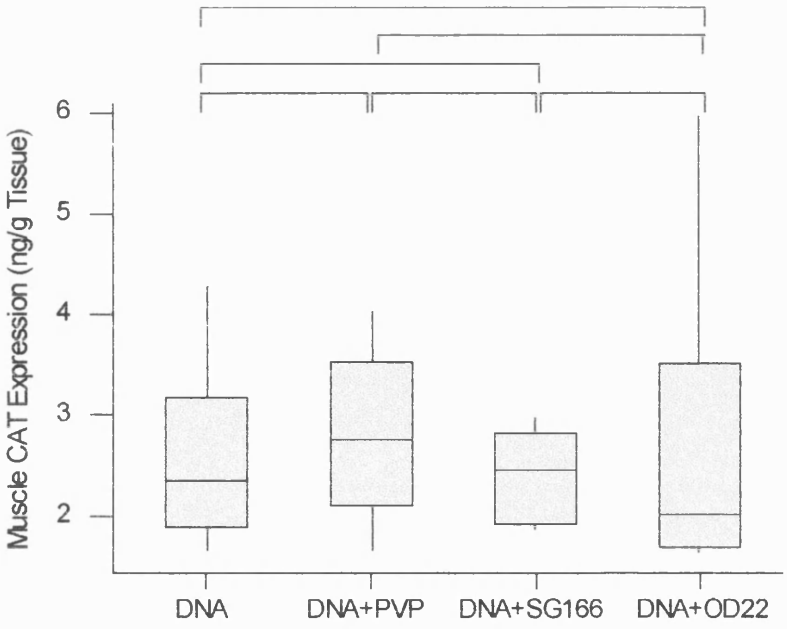


Figure 57 Chloramphenicol acetyl transferase expression in mouse muscle after direct injection of various formulations of pCMVcat. In this graph compound **155** is denoted SG166 and compound **167** is denoted OD22.

Another set of experiments examined compounds **167** and **168** compared to PVP and naked DNA using the pCMVβ-Gal reporter plasmid (figures **58** and **59**). No enhancement of gene expression was seen with **167**, **168** or with PVP in either muscle or tumour.

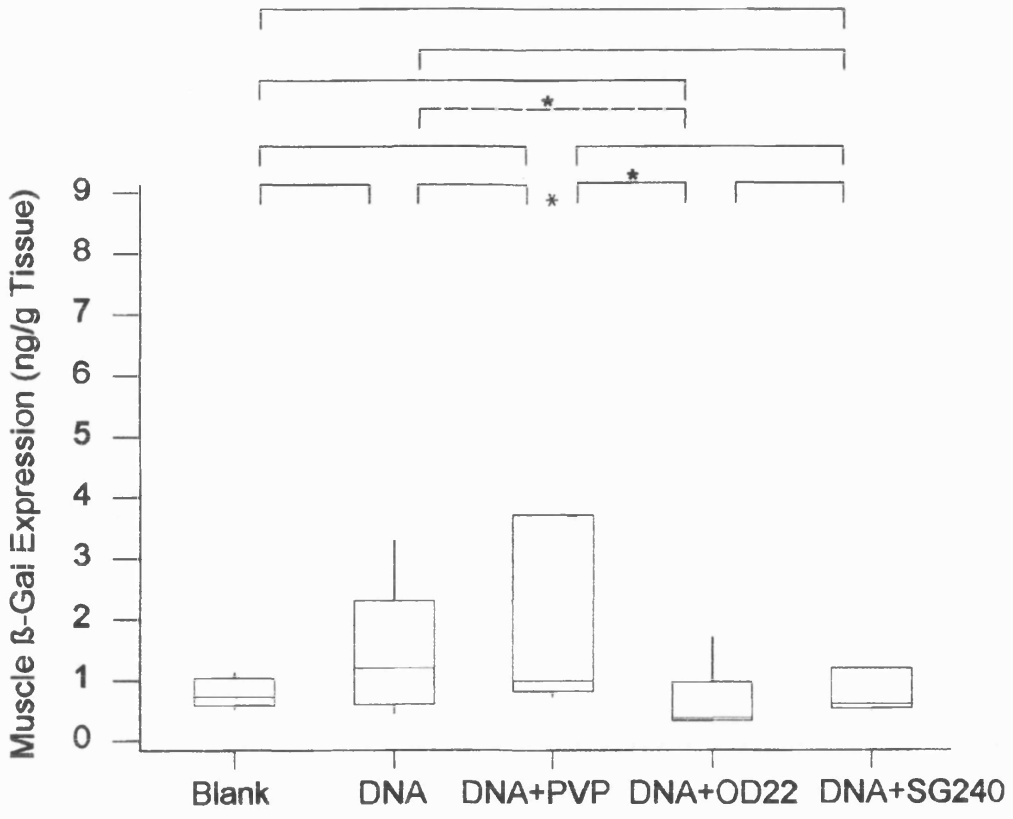


Figure 58 β-Galactosidase expression in mouse muscle after direct injection of various formulations of pCMVβ-Gal. In this graph compound **167** is denoted OD22 and compound **168** is denoted SG240.

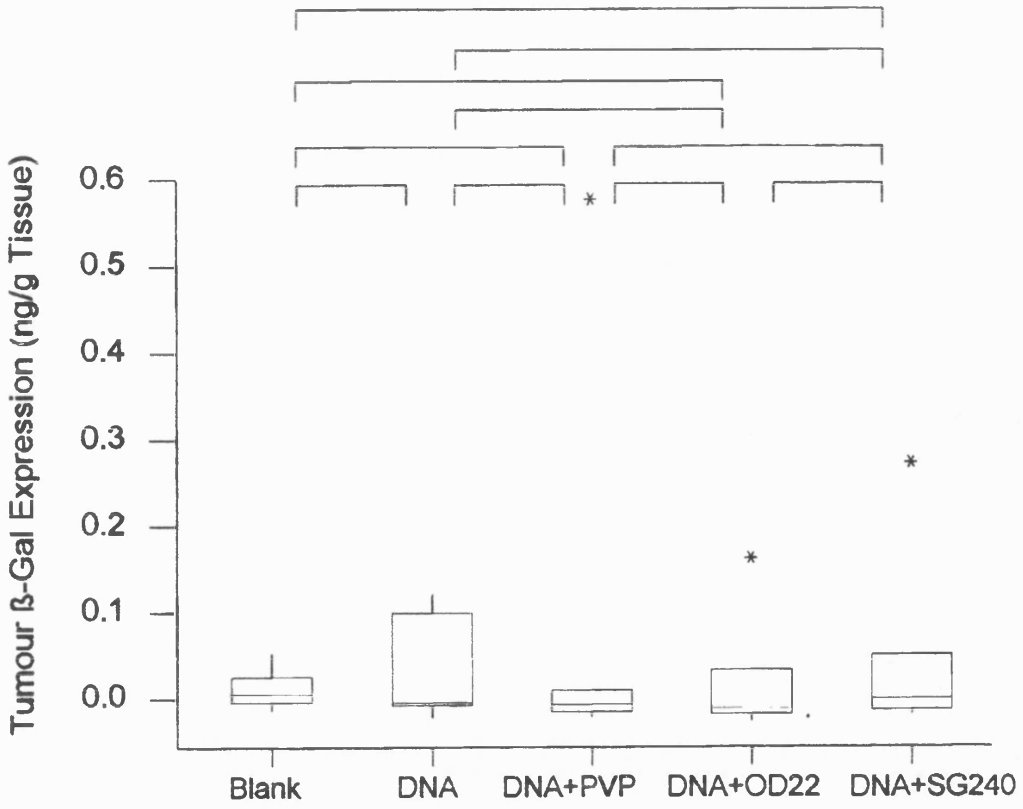


Figure 59 β-Galactosidase expression in tumour after direct injection of various formulations of pCMVβ-Gal. In this graph compound **167** is denoted OD22 and compound **168** is denoted SG240.

4 Conclusions

Versatile, efficient syntheses of protected linear tetramines, suitable for coupling to mPEGs, have been demonstrated and N-methylation was achieved in high yield by alkylating trifluoroacetamide derivatives. A high yielding synthesis of a linear hexamine was devised. Acceptable methods for the coupling of mPEG glycidyl ethers to primary and secondary nitrogens of polyamines have been established. The coupling may have room for improvement in terms of yield; relatively low reactivity of the glycidyl ether and difficult chromatography to isolate the conjugates are possibly responsible for this. In one respect the use of N-methylated derivatives was vindicated since diPEGylation of primary amines readily occurred, however, it has been demonstrated that column chromatography can be carried out to separate mono from diPEGylated conjugates, so in this respect the use of N-methyl derivatives is redundant. Exploration of alternative isolation and

purification techniques would have been desirable. The use of higher molecular weight mPEGs may have allowed purification using techniques such as dialysis, gel permeation chromatography and centrifugation but would have caused problems with characterisation of the conjugates; the more extensive use of mPEG 2000 may have been desirable since lower molecular weight PEGs such as 550 may be associated with some toxicity.

The synthesis of two novel branched polyamines was achieved and these were coupled to mPEG 550 using the mPEG 550 chloroformate. The use of the chloroformate has the advantage over the glycidyl ether in that it is considerably more reactive, however, a suitable spacer must be used if the basicity of the conjugating amine is to be retained and it is less stable upon storage.

The conjugates show a range of interactions with DNA and this work essentially suggests that the higher the degree of PEGylation or the higher the PEG molecular weight, the weaker the interaction with DNA. This may be because of steric effects; the PEG section of one conjugate molecule, when complexed, may prevent other conjugates from accessing the DNA. Further, given the highly hydrophilic nature of PEG, it may be more favourable for the conjugate to remain in free solution rather than forming strong complexes with DNA. Increasing the number of nitrogens enhances DNA interaction, this predictable effect was shown by the synthesis of the hexamine and branched pentamine conjugates (chapter 7).

Although the interaction of these conjugates with DNA is relatively weak, it may be enough to protect plasmids from nucleases and aid dispersion with tissues such as muscle and tumour *i.e.* these conjugates may find use as PINC systems. As judged by the ethidium bromide assays it is unlikely that any of these conjugates cause DNA to condense with the possible exception of the monoPEGylated hexamine **167** which caused a reduction in ethidium bromide-DNA complex fluorescence of *ca.* 65% suggesting a large conformational change in the DNA. Further studies using different techniques are required to determine the nature of the complex between DNA and these novel copolymers.

The *in vivo* experiments show that the conjugates tested show a similar enhancement of gene expression to PVP. Compounds **155** and **167** show a significant enhancement of pCMVcat expression in RIF-1 tumours compared to a formulation of DNA in isotonic saline alone. There appears to be no advantage gained over PVP or **155** by using **167**, the monoPEGylated hexamine which showed the strongest DNA binding of all the conjugates synthesised. Although these results provide useful information for the future development of PINC systems the levels of expression

gained in muscle were somewhat disappointing. However the level of gene expression obtained in muscle can be highly variable and be strongly influenced by injection technique [Mumper and Rolland, 1998].

In summary, the second part of the thesis described the synthesis a number of mPEG-polyamine conjugates demonstrating a range of affinities for DNA by ethidium bromide assays; two of the conjugates enhanced the tumoural expression of reporter genes in mice bearing RIF-1 tumours when formulated with plasmid DNA and injected intratumourally.

Chapter 9:

Experimental

1. Materials and General Procedures

Chemicals were purchased from the Aldrich, Fluka, Acros, Lancaster or Sigma chemical companies. Anhydrous solvents were purchased in anhydrous form, except THF which was used freshly distilled from Na/benzophenone. ^{1}H, ^{13}C and ^{19}F NMR spectra were recorded on either JEOL GX270 or EX400 spectrometers. ^{31}P NMR spectra were recorded on JEOL FX-90Q or EX-400 spectrometers. Chemical shifts are reported in ppm relative to standards. The standards were TMS for samples in $CDCl_3$ and $[^{2}H]_7$-DMF, TMS or DMSO for samples in $[^{2}H]_6$-DMSO, TSP or HDO for samples in D_2O, external 85 % aqueous phosphoric acid for ^{31}P spectra and CCl_3F for ^{19}F spectra. Multiplicities are indicated as follows: s (singlet), d (doublet), t (triplet), dd (doublet of doublets), q (quartet), m (multiplet), td (triplet of doublets), qn (quintet), tt (triplet of triplets) and br (broad signal). Thin layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminium sheets silica 60 F_{254}, Art no. 5554). The spots were visualised using phosphomolybdic acid, potassium permanganate, ninhydrin, iodine or UV light. Melting points were determined using a Reichert-Jung Thermo Galen Kofler block and are uncorrected. Elemental analysis was carried out by the University of Bath microanalysis service. Optical rotations were observed on an Optical Activity Ltd AA-10 polarimeter. Mass spectra were obtained by chemical ionisation (CI), by electrospray (ES) or by fast atom bombardment (FAB) in a VG707E spectrometer and were recorded by the University of Bath mass spectrometry service. MALDI-TOF mass spectra were recorded at The School of Pharmacy, University of London. All evaporations were carried out under reduced pressure. Chromatography refers to the procedure developed by Clark Still [Clark Still *et al*, 1978] and was carried out using silica gel (Merck Kieselgel 60, mesh size 0.040-0.063mm). Ion-exchange chromatography was carried out on an LKB-Pharmacia medium pressure ion-exchange chromatograph on Q sepharose fast flow resin. The brine was saturated. HCl was generated by addition of concentrated sulfuric acid to NaCl. The boiling points quoted refer to the oven temperature of the kugelrohr apparatus.

2 Modification of Briggs' Phosphate Assay

Qualitative test

250µl aliquots of each ion-exchange column fraction were transferred to test tubes and 4 drops of concentrated H_2SO_4 were added. The samples were heated at 150-200°C for one hour. The

samples were then cooled and 250µl water was added to each, followed by: 500µl aqueous ammonium molybdate solution (prepared using 2.5g in 20ml water and 8ml concentrated H_2SO_4); 250µl aqueous hydroquinone solution (100mg in 20ml water and 1 drop concentrated H_2SO_4); 250µl aqueous sodium sulfite solution (4g in 20ml water). Each sample was boiled for 8-10 seconds using a heat gun and then allowed to cool. The phosphate containing fractions could be identified by their blue colour.

Quantitative test

The qualitative assay was repeated but this time known concentrations of potassium dihydrogen phosphate were co-assayed in order to construct a standard curve. Eight different concentrations of standard were used in triplicate. Three different sample sizes of the inositol phosphate to be analysed were taken, again in triplicate. The sample volumes taken were of an appropriate size such that they would lie within the limits of the standard curve, based on an assumed yield of around 70-80% at the phosphate deblocking step.

On completion of the procedure as described for the qualitative assay, the test-tube fractions were transferred to 10ml volumetric flasks, water was added to 10ml and the U.V. absorbance at 340nm was measured in a 3ml quartz cell. This allowed the construction of a standard curve, an example of which is shown in figure **60**, the concentration of the unknown samples could thus be calculated.

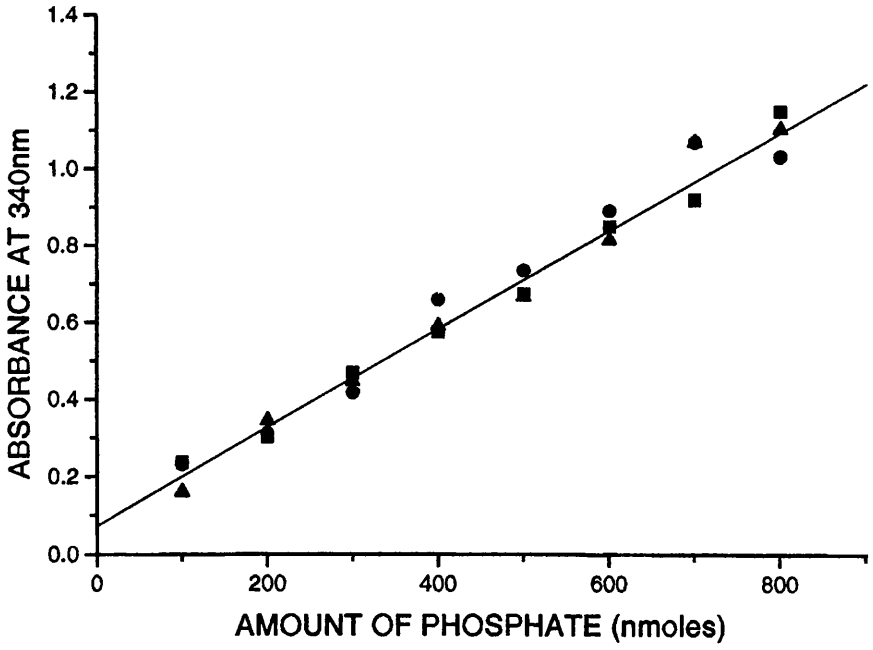


Figure 60 Standard curve for Brigg's phosphate assay.

3 Ethidium Bromide Assay

Calf thymus DNA "highly polymerised" sodium salt and poly-L-lysine hydrobromide (30000-70000Da) were purchased from Sigma. Plasmid DNA (pCMVcat) and PVP molecular weight 50000Da were generous gifts from GeneMedicine, The Woodlands, Texas, USA. The experiments were performed in HEPES buffered saline (HBS), containing 20mM HEPES and 150mM NaCl; the pH was adjusted to 7.4 with 1M aqueous NaOH. The PVP experiment was performed in 0.1M acetate buffer pH 4 (41ml 0.2M acetic acid + 9ml 0.2M potassium acetate, water to 100ml). The experiment at pH 5.5 was performed in MES buffered saline, containing 20mM MES and 150mM NaCl; the pH was adjusted to 5.5 with 1M hydrochloric acid. Fluorescence was monitored on a Perkin Elmer Luminescence Spectrometer (LS-50B) with an excitation wavelength of 260nm and an emission wavelength of 600nm.

Assay procedure

To 6μg of DNA in HBS (3ml) is added 3μl of ethidium bromide solution 0.5mg/ml and the mixture is stirred in the fluorimeter cuvette until no change in fluorescence occurred (usually *ca.* 2-3 minutes). An aliquot of compound to be tested (dissolved in HBS), typically 4μl, is added with continuous stirring. The new fluorescence reading is taken 45-60 seconds later then a another aliquot of compound is added. This is repeated until no further change in fluorescence is observed or when the volume of polymer added is 2.5% of the total volume of the system.

The percent relative fluorescence intensity was determined using the following equation:

$$F_r = (F_{obs} - F_e) / (F_0 - F_e)$$

Where F_r is the relative fluorescence, F_{obs} is the measured fluorescence, F_e is the fluorescence of EB without DNA and F_0 is the initial fluorescence of the EB-DNA complex before any polymer is added.

4 D-*myo*-Inositol 1,4,5-Trisphosphate

myo-Inositol orthoacetate (62)

To *myo*-inositol (20.0 g, 0.11 mol) and PTSA (5.3g, 0.03 mol) in DMF (200 ml) was added triethyl orthoacetate (38 ml, 0.21 mol) slowly at 140°C under N_2. The mixture was stirred for 2h and allowed to cool. The DMF was evaporated and saturated aqueous $NaHCO_3$ (40 ml) was added. The mixture was stirred for 10 min, filtered, then diluted with water (200 ml). The solution was washed with $CHCl_3$ (3× 100 ml) and the water was evaporated. The residue, in MeOH (800 ml), was heated to 50°C and filtered. Evaporation, chromatography (acetonitrile) and recrystallisation (MeOH) gave *myo*-inositol orthoacetate (12.95g, 57%) as colourless crystals: mp 185-187°C (with softening at 165°C); Found: C, 47.0; H, 6.0. calc. for $C_8H_{12}O_6$: C, 47.06; H, 5.92%; δ_H (400 MHz; $[^2H]_6$-DMSO) 1.28 (3H, s, CH_3), 3.94-4.0 (4H, m, Ins H), 4.21-4.27 (2H, m, Ins H), 5.19 (1 H, s, OH-2), 5.38 (2H, d, J 5.2 Hz, OH-4 and OH-6); δ_C (100 MHz, $[^2H]_6$-DMSO) 24.30 (CH_3), 57.70, 67.24, 69.24, 74.98 (Ins C), 107.66 (CCH_3); *m/z* (FAB+) 205 [$(M+H)^+$, 100%].

Dicamphanate esters of myo-inositol orthoacetate

In dichloromethane

To *myo*-inositol orthoacetate (1.00g, 4.90mmol), triethylamine (2ml) and DMAP (100mg) in dry CH_2Cl_2 (40ml) was added (–)-camphanoyl chloride (2.25g, 10.4 mmol), in dry CH_2Cl_2 (10ml) slowly at 0°C under N_2. After 15 min, the mixture was allowed to reach room temperature and stirring was continued for a further 2h. Evaporation and chromatography (CH_2Cl_2/EtOAc 3:1) gave the 2,6-dicamphanate **59** (940mg, 34%) as a white solid and the 2,4-dicamphanate **63** (677mg, 24%) as a white solid. Samples for analysis were recrystallised from EtOAc/hexane.

In pyridine

To a solution of *myo*-inositol orthoacetate (970mg, 4.75 mmol) in dry pyridine (10ml) was added DMAP (50mg) and (–)-camphanoyl chloride (2.60g, 12.0mM) at 0°C. The mixture was allowed to reach room temperature and was stirred for 1h. The solvents were evaporated. The residue, in CH_2Cl_2, was washed with 1M hydrochloric acid (50ml) and brine (70ml), then dried with $MgSO_4$. Evaporation and chromatography gave the 2,6-dicamphanate **59** (542mg, 20%) as a white solid and the 2,4-dicamphanate **63** (996mg, 37%) as a white solid.

1D-2,6-Di-*O*-(–)-camphanoyl *myo*-inositol orthoacetate (59)

mp 228-231°C; $[\alpha]_D^{24}$ +17° (c 1.0, CH_2Cl_2); Found: C, 59.6; H, 6.45. calc. for $C_{28}H_{36}O_{12}$: C, 59.59; H, 6.43%; δ_H (400 MHz; $CDCl_3$) 0.98, 0.99, 1.08, 1.09, 1.10, 1.11 (18H, 6× s, Camph

CH_3), 1.44 (3H, s, O_3CCH_3), 1.67-1.76 (2H, m, Camph CH_2), 1.91-2.10 (4H, m, Camph CH_2), 2.39-2.53 (2H, m, Camph CH_2), 3.23 (1H, d, J 6.4 Hz, OH-2), 4.33-4.36 (1H, m, H-3), 4.39-4.43 (1H, m, H-1), 4.45-4.50 (1H, m, H-5), 4.57-4.62 (1H, m, H-4), 5.21-5.25 (1H, m, H-2), 5.52-5.57 (1H, m, H-6); δ_C (100 MHz; $CDCl_3$) 9.59, 9.66, 16.47, 16.54, 16.65 (Camph CH_3), 23.94 (O_3CCH_3), 28.76, 28.90, 30.44, 30.86 (Camph CH_2), 54.41, 54.51, 54.80, 54.89, 90.86, 91.03 (Camph C), 63.72, 66.78, 68.15, 69.14, 69.30, 71.86 (Ins C), 108.8 (O_3C), 166.15, 166.81, 177.73, 178.02 (Camph CO); *m/z* (FAB+) 565 [$(M+H)^+$, 100%].

1D-2,4-Di-*O*-(−)-camphanoyl *myo*-inositol orthoacetate (63)

mp 201-204°C; $[\alpha]_D^{24}$ −4° (c 1.0, CH_2Cl_2); Found: C, 59.5; H, 6.5. calc. for $C_{28}H_{36}O_{12}$: C, 59.59; H, 6.43%; δ_H (400MHz, $CDCl_3$) 0.96, 0.97, 1.09, 1.10, 1.12, 1.14 (18H, 6× s, Camph CH_3), 1.43 (3H, s, O_3CCH_3), 1.63-1.74 (2H, m, Camph CH_2), 1.90-2.11 (4H, m, Camph CH_2), 2.43-2.53 (2H, m, Camph CH_2), 3.37 (1H, d, J 6.1Hz, OH-6), 4.30-4.36 (1H, m, H-1), 4.40-4.45 (1H, m, H-5), 4.49-4.55 (1H, m, H-3 or H-6), 4.51-4.62 (1H, m, H-3 or H-6), 5.23-5.25 (1H, m, H-2), 5.53-5.55 (1H, m, H-4); δ_C (100MHz, $CDCl_3$) 9.58, 9.63, 16.50, 16.58, 16.63, 16.81 (Camph CH_3), 24.0 (O_3CCH_3), 28.83, 29.03, 30.57, 30.70 (Camph CH_2), 54.31, 54.51, 54.78, 54.89, 91.01, 91.12 (Camph C), 63.74, 66.67, 68.08, 68.96, 71.93 (Ins C), 108.79 (O_3CCH_3), 165.94, 166.94, 177.86, 177.90 (CO).

1D-2,4,6-Tri-*O*-(−)-camphanoyl *myo*-inositol orthoacetate (64)

mp 210-211°C; Found: C, 61.2; H, 6.43. calc. for $C_{38}H_{48}O_{15}$: C, 61.28; H, 6.50%; δ_H (400MHz, $CDCl_3$), 0.98, 1.02, 1.11, 1.13, 1.14, 1.17 (27H, 6× s, Camph CH_3), 1.53 (3H, s, O_3CCH_3), 1.73-1.80 (4H, m, Camph CH_2), 1.98-2.08 (3H, m, Camph CH_2), 2.12-2.19 (1H, m, Camph CH_2), 2.30-2.38 (2H, m, Camph CH_2), 2.48-2.60 (2H, m, Camph CH_2), 4.36-4.39 (1H, m, Ins H), 4.53-4.58 (2H, m, Ins H), 5.05-5.09 (1H, m, H-2), 5.70-5.72 (1H, m, H-4 or H-6), 5.77-5.79 (1H, m, H-4 or H-6).

1D-2,6-Di-*O*-(−)-camphanoyl *myo*-inositol (51)

A solution of the alcohol **59** (455mg, 0.81mmol) in MeOH (45ml) and 5M Hydrochloric acid (2ml) was heated to reflux. After 5h, further 5M Hydrochloric acid (2ml) was added and heating to reflux continued for 9h. Evaporation then chromatography ($CHCl_3$/MeOH 5:1) gave tetrol **51** (40mg, 9%) as a white solid: mp 212-215°C (lit. [Riley, 1996] mp 211-213°C); $[\alpha]_D^{25}$ −8° (c 0.01, DMF) {lit. [Riley, 1996] $[\alpha]_D^{25}$ −8° (c 0.01, DMF)}; δ_H (400 MHz; $[^2H]_6$-DMSO) 0.86-1.07 (18H, m, Camph CH_3), 1.52-1.61 (2H, m, Camph CH_2), 1.87-2.05 (4H, m, Camph CH_2), 2.35-2.50 (2H, m, Camph CH_2), 3.22-3.28 (1H, m, D_2O ex. gives t, J 9.2Hz, H-4), 3.44-3.46 (1H, m, D_2O ex. gives t, J 9.5Hz, H-5), 3.50-3.54 (1H, m, D_2O ex. gives dd, J 2.8, 9.8Hz, H-3), 3.74-3.79 (1H, m, D_2O ex. gives dd, J 2.8, 10.1Hz, H-1), 4.98-5.03(1H, t, J 10.3Hz, H-6),

5.09 (1H, d, J 4.4Hz, OH-5), 5.16 (2H, d, J 5.4Hz, OH-3 and OH-4), 5.32 (1H, d, J 4.9Hz, OH-1), 5.37 (1H, t, J 2.4Hz, H-2); δ_C (100MHz; $[^2H]_6$-DMSO) 9.51, 9.54, 16.06, 16.29, 16.33, 16.49 (Camph CH_3), 28.33, 28.38, 30.26, 30.19 (Camph CH_2), 53.89, 53.95, 54.28, 54.35, 91.09, 91,29 (Camph C), 66.89, 68.82, 71.96, 73.37, 75.75, 76.19 (Ins C), 166.16, 178.01 (Camph CO).

Acetate esters of 1D-2,6-di-*O*-(−)- camphanoyl *myo*-inositol

The alcohol **59** (870mg, 1.54mmol) in 80% aqueous trifluoroacetic acid (60ml) was stirred at room temperature for 7 days. Evaporation then chromatography (EtOAc) gave the 3-acetate **49** (172mg, 20%) as a white solid, the 1-acetate **60** (227mg, 25%) as a white solid, and the tetrol **51** (113mg, 14%).

1D-3-Acetyl-2,6-di-*O*-(−)-camphanoyl *myo*-inositol (49)

mp 226-229°C; $[\alpha]_D^{25}$ +5.2° (c 1.9, DMF); δ_H (400 MHz; $[^2H]_7$-DMF) 0.96, 1.02, 1.05, 1.10, 1.12, 1.17 (18H, 6× s, Camph CH_3), 1.55-1.67 (2H, m, Camph CH_2), 1.94-2.14 (4H, m, Camph CH_2), 2.02 (3H, s, $COCH_3$), 2.49-2.61 (2H, m, Camph CH_2), 3.61 (1H, dt, J 5.9, 9.3Hz, D_2O ex. gives t, J 9.8Hz, H-5), 3.86 (1H, dt, J 4.9, 9.8Hz, D_2O ex. gives t, J 9.8Hz, H-4), 4.15-4.17 (1H, m, D_2O ex. gives dd, J 2.8, 10.4Hz, H-1), 4.96 (1H, dd, J 2.8, 10.1Hz, H-3), 5.29 (1H, t, J 10.1Hz, H-6) 5.58 (1H, d, J 5.4Hz, OH-5), 5.60 (1H, m, OH-4), 5.62-5.63 (1H, m, D_2O ex. gives t, J 2.8Hz, H-2), 5.75 (1H, d, J 5.9Hz, OH-1); *m/z* (FAB+) 583.2394. $(M+H)^+$ ($C_{28}H_{39}O_{13}$ requires 583.2391).

1D-1-Acetyl-2,6-di-*O*-(−)-camphanoyl *myo*-inositol (60)

mp 146-150°C (with softening at *ca.*112°C); δ_H (400MHz, $CDCl_3$), 0.90, 1.05, 1.07 (9H, 3× s, CamphCH_3), 1.09 (6H, s, Camph CH_3), 1.13 (3H, s, CamphCH_3), 1.65-1.76 (2H, m, Camph-CH_2), 1.92-2.10 (3H, m, Camph CH_2), 1.96 (3H, s, $COCH_3$), 2.11-2.18 (1H, m, Camph CH_2), 2.38-2.53 (2H, m, Camph CH_2), 3.67 (1H, t, J 9.3Hz, H-5), 3.90-3.95 (2H, m, H-3 and H-4), 4.35 (3H, br s, OH), 5.09 (1H, dd, J 2.4, 10.7Hz, H-1), 5.36 (1H, t, J 10.3Hz H-6), 5.62-5.65 (1H, m, H-2); *m/z* (FAB+) 583.2418 $(M+H)^+$ ($C_{28}H_{39}O_{13}$ requires 583.2391); 584.2444 $(M+H)^+$ ($^{13}C_1C_{27}H_{39}O_{13}$ requires 584.2424).

1D-3-Acetyl-2,6-di-*O*-(−)-camphanoyl-*myo*-inositol-1,4,5-tris(dibenzyl phosphate) (66)

To bis(benzyloxy)diisopropylaminophosphine (174mg, 5.03mmol) in dry CH_2Cl_2 (3ml) was added 1*H*-tetrazole (70mg, 1.0mmol). The mixture was stirred at room temperature for 30 min then added to the triol **49** (65mg, 0.112mmol) with further dry CH_2Cl_2 (1ml). The mixture was stirred at room temperature for 30 min [^{31}P NMR (90 MHz) showed phosphite triester signals

at 142.1, d, J 7.3Hz; 141.5, d, J 7.3Hz; and 140.7, s]. The mixture was cooled to -78°C and MCPBA (57-86%, 115mg) was added. The mixture was allowed to reach room temperature and was stirred for 20min. CH_2Cl_2 (50ml) was added and this solution was washed with 10% aqueous Na_2SO_3 (50ml), saturated aqueous $NaHCO_3$ (50ml) and brine (50ml). The organic layer was dried with $MgSO_4$. Evaporation and chromatography (EtOAc/hexane 3:2) gave the title compound (110mg, 72%) as a colourless oil: δ_P (162 MHz $CDCl_3$) −2.05, −2.18, −2.33; δ_H (400 MHz $CDCl_3$;) 0.92, 0.95, 1.00, 1.04, 1.09, 1.11 (18H, 6× s, camph CH_3), 1.47-1.54 (1H, m, camph CH_2), 1.62-1.69 (1H, m, camph CH_2), 1.71-1.75 (1H, m, camph CH_2), 1.78 (3H, s, $COCH_3$), 1.88-1.96 (2H, m, camph CH_2), 2.04-2.11 (1H, m, camph CH_2), 2.24-2.31 (1H, m, camph CH_2), 2.37-2.44 (1H, m, camph CH_2), 4.22 (1H, m, H-1), 4.40 (1H q, J 9.8Hz, H-4 or H-5), 4.83-5.08 (14H, m, 6× $PhCH_2$ and H-3 and H-4 or H-5), 5.61 (1H, t, J 9.8Hz, H-6), 5.93 (1H, t, J 2.7Hz, H-2), 7.20-7.37 (30H, m, Ph); *m/z* (FAB+) 1365.4273 $(M+H)^+$ ($^{13}C_2C_{68}H_{78}O_{22}P_3$ requires 1365.4265); 1364.4221 $(M+H)^+$ ($^{13}C_1C_{69}H_{78}O_{22}P_3$ requires 1364.4231); 1363.4216 $(M+H)^+$ ($C_{70}H_{78}O_{22}P_3$ requires 1363.4198).

D-*myo*-Inositol-1,4,5-trisphosphate (19)

To the tris(dibenzylphosphate) **66** (110mg, 0.081mmol) in MeOH (20ml) was added 10% Pd on activated charcoal (100mg) as a slurry in water (1ml). The mixture was stirred vigorously for 12h at room temperature under H_2. The catalyst was filtered off and washed with MeOH (15ml). Evaporation of the solvent from the filtrate and washings gave a glassy white solid. This was dissolved in concentrated aqueous ammonia (10ml) and transferred to a sealed bottle and heated at 60°C for 4h. The solvents were evaporated and the residue was purified by ion exchange chromatography on Q Sepharose fast flow resin using a gradient of triethylammonium hydrogencarbonate buffer (0-100% 1M). Evaporation gave D-*myo*-inositol-1,4,5-trisphosphate **19**, triethylammonium salt (56μmol, 70%) as a colourless glass: $[\alpha]_D^{23}$ −17.0 (c 0.024, triethylammonium hydrogencarbonate buffer, pH 7.6) {lit. (ammonium salt)[Ozaki *et al*, 1992] $[\alpha]_D^{23}$ −10.3° (c 1.80, water, pH6.73)}; δ_P (162 MHz, D_2O) −0.40, 0.34, 0.92; δ_H (400 MHz, D_2O) 3.59 (1H, dd, J 2.8, 9.8Hz, H-3), 3.77 (1H, t, J 9.5, H-6), 3.86-3.94 (2H, m, H-5 and H-1), 4.13-4.20 (2H, m, H-4 and H-2); *m/z* (FAB−) 418.9552 $(M-H)^-$ ($C_6H_{14}O_{15}P_3$ requires 418.9546).

5 DL-(1,3,5/2,4,6)-6-hydroxymethyl-cyclohexane-1,2,3,4,5-pentol-1,2,4,7-tetrakisphosphate

1,3,5-*O*-Methylidine-*myo*-inositol (74)

To *myo*-inositol (20.0g, 0.11mol) and PTSA (4.7g, 0.025mol) in DMF (200ml) was added triethyl orthoformate (35ml, 0.210mol) slowly at 140°C. The mixture was stirred for 3h, allowed to cool and the DMF was evaporated. 10% Aqueous Na_2CO_3 solution (50ml) was added. The mixture was stirred for 15 minutes, filtered then diluted with water (200ml), the solution was washed with $CHCl_3$ (3× 100ml) and the water was evaporated. The residue, in MeOH (600ml), was heated to 50°C and the remaining solid was filtered off. Evaporation, chromatography (acetonitrile) and recrystallisation (MeOH) gave 1,3,5-*O*-methylidine-*myo*-inositol **74** (12.1g, 57%) as colourless crystals: mp >275°C (decomposes) (lit. [Billington *et al*, 1989] mp 300-302°C); Found: C, 44.3; H, 5.3. calc. for $C_7H_{10}O_6$: C, 44.22; H, 5.30%; δ_H (270MHz; $[^2H]_6$-DMSO) 3.94-3.96 (2H, m, H-1 and H-3), 3.99 (1H, br s, H-2), 4.06 (1H, m, H-5), 4.26-4.29 (2H, m, H-4 and H-6), 5.33 (1H, br s, OH-2), 5.45 (1H, s, O_3CH) 5.47 (2H, br s, OH-4 and OH-6).

2,6-Di-*O*-(4-methoxybenzyl)-1,3,5-*O*-methylidene-*myo*-inositol (75)

To *myo*-inositol orthoformate **74** (11.68g, 61.5mmol) in dry DMF (250ml) was added NaH (60% dispersion in mineral oil, 5.4g, 141mmol) slowly at room temperature. The mixture was stirred for 1h then 4-methoxybenzyl chloride (17.5ml, 129mmol) was added. After 3h water (50ml) was added slowly, then the solvents were evaporated. Water (300ml) was added to the residue and the products were extracted with CH_2Cl_2 (3× 200ml). The pooled organic phases were washed with brine and then dried with $MgSO_4$. Evaporation, chromatography (CH_2Cl_2/EtOAc 5:1) and recrystallisation (EtOAc/hexane) gave the title compound (10.26g, 38%) as colourless crystals: mp 120-121°C (lit. [Riley *et al*, 1998] mp 120-121°C); Found: C, 64.1; H, 6.1. calc. for $C_{23}H_{26}O_8$: C, 64.18; H, 6.09%; δ_H (270MHz; $CDCl_3$;) 3.25 (1H, d, J 11.4Hz, OH-2), 3.78 (6H, s, OCH_3), 4.13-4.15 (1H, m, H-2), 4.17-4.21 (2H, m, H-1 and H-3), 4.33 (2H, t, J 3.5Hz, H-4 and H-6), 4.39-4.41 (1H, m, H-5), 4.48 (2H, d, J 11.0Hz, CH_2), 4.56 (2H, d, J 11.0Hz, CH_2), 5.46 (1H, s, O_3CH), 6.69-6.83 (4H, m, Ph), 7.15-7.19 (4H, m, Ph); δ_C (68 MHz; $CDCl_3$) 55.14 (OCH_3), 61.35 (Ins C), 67.77 (Ins C), 71.26 (CH_2), 72.95 (Ins C), 73.40 (Ins C), 103.21 (O_3C), 113.78 (Ph C), 129.30 (Ph C), 129.54 (Ph C), 159.32 ($COCH_3$).

2,4,6/3,5-Pentahydroxy-3,5-di-*O*-(4-methoxybenzyl)-2,4,6-*O*-methylidene-cyclohexanone (76)

To oxalyl chloride (12.8ml of a 2.0M solution in CH_2Cl_2, 25.6mmol) in dry CH_2Cl_2 (40ml) was added dry DMSO (3.6ml, 51mmol) in dry CH_2Cl_2 (5ml) dropwise at –60°C under N_2. The mixture was stirred for 10min at –60°C. The alcohol **75** (10.00g, 23.2mmol) in dry CH_2Cl_2 (30ml) was added dropwise. The mixture was stirred for 30min at –60°C, then triethylamine (15ml) was added. The mixture was allowed to warm to room temperature, then water (100ml) was added and the product extracted with CH_2Cl_2 (2× 150ml). The organic layer was washed with brine (200ml), 1% hydrochloric acid (150ml), water (150ml), 10% aqueous $NaHCO_3$ solution (200ml), and water (200ml), then dried with $MgSO_4$. Evaporation gave a white solid which was dissolved in toluene (300ml) and heated to reflux with azeotropic removal of water using a Dean and Stark trap for 3h. Evaporation then recrystallisation (EtOAc/hexane) under N_2 gave the ketone **76** (8.13g, 82%) as colourless crystals: mp 126-127°C (lit. [Riley *et al*, 1998] mp 125-126°C); IR 1760cm^{-1} CO; δ_H (400MHz; $CDCl_3$) 3.78 (6H, s, OCH_3), 4.39 (2H, dd, J 2.5Hz, 1.5Hz, H-3 and H-5), 4.48-4.50 (1H, m, H-4), 4.51-4.54 (6H, m, CH_2, H-2 and H-6), 5.63 (1H s O_3CH), 6.80-6.84 (4H m, Ph), 7.14-7.18 (4H, m, Ph); δ_C (100MHz; $CDCl_3$) 55.23 (CH_3), 68.87 (Ins C), 71.17 (CH_2) 76.35 and 77.98 (Ins C), 102.63 (O_3CH), 113.82 and 129.51 (Ph C), 128.96 (CCH_2), 159.45 (PhCOCH_3), 199.28 (CO).

2,4-Di-*O*-(4-methoxybenzyl)-6-methylidine-1,3,5-*O*-methylidine-cyclohexane 1,3,5/2,4-pentol (77)

To methyltriphenylphosphonium bromide (12.4g, 34.7mmol) in dry THF (40ml) was added potassium *tert*-butoxide (33ml of a 1M solution in THF, 33mmol) at 0°C under N_2, with stirring. The yellow suspension was allowed to reach room temperature. After 10min, the ketone **76** (7.01g, 16.4 mmol) in dry THF (60ml) was added. The mixture was heated to reflux for 3h. The THF was evaporated and the residue was dissolved in diethyl ether (200ml), washed with brine (200ml) then dried with $MgSO_4$. Evaporation, then chromatography (EtOAc/hexane 1:2) gave the alkene **77** (5.89g, 84%) as a white solid: mp 90-91°C (from EtOH) (lit. [Riley *et al*, 1998] mp 95-97°C); Found: C, 67.5; H, 6.2 calc. for $C_{24}H_{26}O_7$: C, 67.59; H, 6.15%; δ_H (270MHz; $CDCl_3$) 3.80 (6H, s, CH_3), 4.23 (2H, dd, J 3.5, 3.6Hz, H-2 and H-4), 4.30-4.35 (1H, m, H-3), 4.40-4.42 (2H, m, H-1 and H-5), 4.53 (2H, d, J 11.9Hz, CH_2), 4.58 (2H, d, J 11.9Hz, CH_2), 5.25 (2H, s, $=CH_2$), 5.56 (H, s, OC_3H), 6.82-6.85 (4H, m, Ph), 7.22-7.25 (4H, m, Ph); δ_C (68.8MHz; $CDCl_3$) 55.17 (OCH_3), 68.97 (Ins C), 71.09 (CH_2), 74.29 and 73.58(Ins C), 103.69 (O_3CH), 113.82, 129.47, 137.19 and 159.40 (Ph C), 114.29 ($=CH_2$), 129.83 (Ins C=); *m/z* 427 [$(M+H)^+$, 100%].

(1,3,5/2,4,6)-6-Hydroxymethyl-1,3,5-*O*-methylidene-2,4-di-*O*-(4-methoxybenzyl)-cyclohexane 1,2,3,4,5-pentol (78)

To the alkene **77** (5.00g, 11.72mmol) was added 9-borabicyclononane (50ml of a 0.5M solution in THF, 25mmol) at room temperature under N_2. The mixture was stirred for 2h at 50°C under N_2. The mixture was then cooled to 0°C, then ethanol (17ml), 6M aqueous NaOH (4.2ml) and 30% H_2O_2 were added dropwise. The mixture was then stirred at 50°C for a further 30 minutes. The mixture was cooled and water (20ml) was added. The aqueous layer was saturated with K_2CO_3 and the organic layer was removed, dried with $MgSO_4$ then evaporated. Chromatography (EtOAc/hexane 1:1) gave the title compound (3.88g, 74%) as a white solid: mp 79-81°C (from EtOH) (lit. [Riley *et al*, 1998] mp 81-82°C); δ_H (400MHz; $CDCl_3$) 1.72-1.76 (1H, m, CH_2OH), 2.95-2.99 (1H, m, H-6), 3.79 (6H, s, CH_3), 4.06-4.08 (2H, m, CH_2OH), 4.26-4.28 (2H, m, H-1 and H-5), 4.34-4.36 (2H, m, H-2 and H-4), 4.48 (1H, d, J 10.7Hz, CH_2), 4.49 (1H, d, J 11.3Hz, CH_2), 4.51-4.53 (1H, m, H-3), 4.57 (1H, d, J 11.3Hz, CH_2), 4.58 (1H, d, J 11.0Hz, CH_2), 5.57 (1H, s, O_3CH), 6.79-6.83 (4H, m, Ph), 7.15-7.19 (4H, m, Ph).

(1,3,5/2,4,6)-6-Hydroxymethyl-2,4-di-*O*-(4-methoxybenzyl)-cyclohexane 1,2,3,4,5-pentol (79)

The alcohol **78** (3.88g, 8.73mmol) in MeOH (150ml)/1M hydrochloric acid (15ml) was heated to 50°C. After 40 min, concentrated aqueous ammonia solution (20ml) was added and the mixture stirred at room temperature for 1h. Evaporation then chromatography ($CHCl_3$/MeOH 5:1 then 1:1) gave the title compound (2.81g, 74%) as a white solid: mp 135-136.5 °C (lit. [Riley *et al*, 1998] mp136-137°C); δ_H (270 MHz; $[^2H]_6$-DMSO) 1.25 (1H, tt, J 10.7, 2.0Hz, H-6) 3.09 (2H, dd, J 9.5, 9.2Hz, H-2 and H-4), 3.21 (1H, dt, J 5.5, 9.2Hz, D_2O ex. gives t, J 9.5Hz, H-3), 3.29-3.33 (2H, m, D_2O ex. gives dd, J 10.4, 9.5Hz, H-1 and H-5), 3.68-3.71 (2H, m, CH_2OH), 3.73 (6H, s, OCH_3), 4.27 (1H, t, J 5.2Hz, CH_2OH), 4.72 (4H, s, CH_2), 4.74 (2H, d, J 5.8, OH-1 and OH-5), 4.92 (1H, d, OH-3 J 5.5), 6.85-6.88 (4H, m, Ph) 7.34-7.36 (4H, m, Ph).

DL-(1,3,5/2,4,6)-1,7-*O*-Benzylidene-6-hydroxymethyl-2,4-di-*O*-(4-methoxybenzyl)-cyclohexane-1,2,3,4,5-pentol (82)

To the tetrol **79** (2.78g, 6.39mmol) in DMF (15ml) was added benzaldehyde dimethyl acetal (1.2g, 7.88mmol) and PTSA (50mg). The flask was fitted with a 25cm air condenser connected to a water pump and the solution was stirred at 70°C under reduced pressure. After 50min, the mixture was cooled to room temperature and triethylamine (2ml) was added. After 1h, the solvents were evaporated and the residue dissolved in CH_2Cl_2 then washed with water (80ml), brine (80ml) then dried with $MgSO_4$. Evaporation, then recrystallisation (EtOAc/hexane) gave the title compound (2.76g, 82%) as a white solid: mp 160-161°C (lit. [Riley *et al*, 1998] mp 158-

160°C); δ_H (400 MHz; $CDCl_3$;) 1.93 (1H, dddd, *J* 10.7, 10.7, 10.7, 4.5Hz, H-6), 2.34 (1H, d, *J* 1.8Hz, OH), 2.66 (1H, d, *J* 1.8Hz, OH), 3.23 (1H, ddd, *J* 1.8, 8.9, 11.0Hz, D_2O ex. gives dd, *J* 8.9, 11.0Hz, H-3), 3.33 (1H, dd, *J* 8.9, 11.0Hz, H-4), 3.51-3.71 (4H, m, H-1, H-2, H-5 and CH_2), 3.78 (6H, br s, OCH_3), 4.50 (1H, dd, *J* 4.3, 11.0Hz, CH_2), 4.60 (1H, d, *J* 11.0Hz, CH_2), 4.63 (1H, d, *J* 11.0Hz, CH_2), 4.96 (1H, d, *J* 11.3Hz, CH_2,), 4.97 (1H, d, *J* 11.3Hz, CH_2), 5.52 (1H, s, C*H*Ph), 6.84-6.90 (4H, m, Ph H), 7.24-7.30 (4H, m, Ph H), 7.33-7.52 (5H, m, Ph H).

DL-(1,3,5/2,4,6)-1,3-Di-*O*-benzyl-5,7-*O*-benzylidene-6-hydroxymethyl-2,4-di-*O*-(4-methoxybenzyl)cyclohexane 1,2,3,4,5-pentol (70)

To the diol **82** (100mg, 0.19mmol) in dry DMF (10ml) was added sodium hydride (25mg of a 60% dispersion in mineral oil, 0.625mmol) at room temperature. After 30min, benzyl bromide (0.05ml, 0.46mmol) was added and the mixture was stirred overnight. Water (5ml) was added then the solvents were evaporated. The residue was dissolved in CH_2Cl_2 (20ml) and washed with brine (20ml) then dried with $MgSO_4$. Evaporation gave a white solid which was washed with pentane then recrystallised (EtOH) to give the title compound (121mg, 90%) as white crystals: mp 135-137°C (lit. [Riley *et al*, 1998] mp 135-137°C); δ_H (270MHz; $CDCl_3$) 1.97-2.00 (1H, m, H-6), 3.21-3.25 (1H, m, H-1) 3.43-3.71 (5H, m, H-2, H-3, H-4, H-5, and CH_2 axial), 3.77 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 4.42 (1H, dd, *J* 11.0, 4.4Hz, CH_2 equatorial), 4.50-4.98 (8H, m, $PhCH_2$), 5.48 (1H, s, CHPh), 6.77-6.84 (4H, m, Ph), 7.20-7.51 (19H, m, Ph).

DL-(1,3,5/2,4,6)-1,3-Di-*O*-benzyl-6-hydroxymethylcyclohexane-1,2,3,4,5-pentol (83)

To **70** (400mg, 0.57mmol) in EtOH (20ml) was added 1M hydrochloric acid (10ml). The mixture was heated to reflux for 3h. Evaporation then chromatography ($CHCl_3$/MeOH 9:1) gave the tetrol **83** (152mg, 71%) as a white solid: mp 124-125°C (from EtOAc/hexane); Found: C, 67.1; H, 7.1. calc. for $C_{21}H_{26}O_6$: C, 67.36; H, 7.00%; δ_H (400MHz; $[^2H]_6$-DMSO) 1.26 (1H, m, H-6), 3.02 (1H, t, *J* 9.3Hz, H-3), 3.26-3.37 [3H, m, D_2O ex. gives: 3.21 (1H, t, *J* 9.2Hz, H-4), 3.32 (1H, t, *J* 10.4Hz, H-5), 3.35 (1H, t, *J* 10.1Hz, H-1)], 3.44-3.46 (1H, m, D_2O ex. gives t, *J* 9.2Hz, H-2), 3.67-3.76 (2H, m, CH_2OH), 4.36 (1H, t, *J* 4.4Hz, CH_2OH), 4.59 (1H, d, *J* 11.2Hz, $PhCH_2$), 4.66 (1H, d, *J* 4.9Hz, OH-5), 4.79 (1H, d, *J* 11.7Hz, $PhCH_2$), 4.83 (1H, d, *J* 11.7Hz, $PhCH_2$), 4.88 (1H, d, *J* 4.9Hz, OH-4), 4.91 (1H, d, *J* 11.2Hz, $PhCH_2$), 5.10 (1H, d, *J* 5.8Hz, OH-2), 7.23-7.46 (10H, m, Ph); δ_C (100MHz; $[^2H]_6$-DMSO) 46.60 (Ins C), 56.29 (CH_2OH), 68.97, 76.67, 76.77, 77.80, 83.19 (Ins C), 73.74 ($PhCH_2$), 127.06, 127.19, 127.69, 127.95, 128.17 (Ph-CH), 139.65, 139.84 (Ph C).

DL-(1,3,5/2,4,6)-3,5-Di-*O*-benzyl-6-hydroxymethylcyclohexane-1,2,3,4,5-pentol-1,2,4,7-tetrakis(dibenzyl phosphate) (84)

To a solution of bis(benzyloxy)diisopropylaminophosphine (1.11g, 3.21mmol) in dry CH_2Cl_2 (3ml) was added 1*H*-tetrazole (450mg, 6.42mmol). The mixture was stirred at room temperature for 30min, then the tetrol **83** (150mg, 0.401mmol) was added. After 40min the mixture was cooled to −78°C and MCPBA (57-86%, 1.1g) was added. The mixture was allowed to reach room temperature and stirring was continued for 30min, then CH_2Cl_2 (50ml) was added. The solution was washed with 10% Na_2SO_3 solution (50ml), saturated $NaHCO_3$ solution (50ml) and brine (50ml) then dried with $MgSO_4$. Evaporation then chromatography ($CHCl_3$/acetone 5:1) gave the title compound (410mg, 72%) as a colourless oil: Found: C, 64.8; H, 5.6. calc. for $C_{77}H_{78}O_{18}P_4$: C, 65.34; H, 5.55%; δ_P (162MHz; $CDCl_3$) −0.81, −1.58, −1.85, −2.18; δ_H (400MHz; $CDCl_3$) 2.02-2.06 (1H, m, H-6) 3.51-3.64 (2H, m, C*H*$_2$-OPO(Bn)$_2$), 4.47-4.50 (2H, m, Ins H), 4.59-5.07 (23H, m, Ins H and Ph-CH_2), 6.97-7.40 (50H, m, Ph-H); *m/z* (FAB+) 1415.4204 $(M+H)^+$ ($C_{77}H_{79}O_{18}P_4$ requires 1415.4217); 1416.4235 $(M+H)^+$ ($^{13}C_1C_{76}H_{79}O_{18}P_4$ requires 1416.4250).

DL-(1,3,5/2,4,6)-6-Hydroxymethyl-cyclohexane-1,2,3,4,5 pentol-1,2,4,7-tetrakisphosphate (68)

To **84** (150mg) in MeOH (40ml) and water (10ml) was added 10% palladium on activated charcoal (200mg). The mixture was treated with hydrogen at 40psi at room temperature overnight. The mixture was then filtered through Celite and the solvents were evaporated. The residue was dissolved in water(100ml) and purified by ion-exchange chromatography on Q Sepharose fast flow resin eluting with a gradient of triethylammonium hydrogencarbonate buffer (0-1M). Evaporation gave the tetrakisphosphate **68** (79μmol, 75%) as a colourless glass: δ_P (162MHz; D_2O) 0.64, 0.39, 0.47, 0.62; δ_H (400MHz; D_2O) 1.61 (1H, t, J 10.7Hz, H-6), 3.39 (1H, t, J 9.2Hz, H-3 or H-5), 3.52 (1H, t, J 10.1Hz, H-3 or H-5), 3.81 (1H, q, J 8.9Hz, H-1 or H-2 or H-4), 3.92-3.99 (3H, m, H-1 or H-2 or H-4 and CH_2), 4.09 (1H, q, J 10.1Hz, H-1 or H-2 or H-4); *m/z* (FAB−) 512.9367 $(M-H)^-$ ($C_7H_{17}O_{18}P_4$ requires 512.9365).

6 mPEG-Polyamine Conjugates

N,N′-Bis(2-cyanoethyl)propane-1,3-diamine (115)

To propane-1,3-diamine (10.0g, 0.13mol) in MeOH (15ml) at 0°C was added acrylonitrile (14.3g, 0.27mol) dropwise over 20min. The mixture was stirred for 20min at 0°C, then at room temperature overnight. Evaporation gave N,N′-bis(2-cyanoethyl)propane-1,3-diamine (24.3g, quant.) as a colourless liquid. A sample for elemental analysis was distilled using kugelrohr apparatus: $bp_{0.3}$ 235°C (lit. [Israel *et al*, 1964] $bp_{0.5}$ 176°C); found: C, 60.0; H, 9.0; N, 31.2. calc. for $C_9H_{16}N_4$: C, 59.96; H, 8.94; N, 31.08%; δ_H (400MHz, $CDCl_3$) 1.49 (2H, s, NH), 1.67 (2H, qn, J 6.7Hz, CH_2CH_2N), 2.53 (4H, t, J 6.7Hz, CH_2), 2.73 (4H, t, J 6.4Hz, CH_2), 2.91 (4H, t, J 6.5Hz, CH_2); δ_C (100MHz $CDCl_3$) 18.67, 29.77, 45.08, 47.55 (CH_2), 118.98 (CN).

N,N′-Bis(3-aminopropyl)propane-1,3-diamine (92)

To N,N′-bis(2-cyanoethyl)propane-1,3-diamine (2.00g, 11.1mmol) in MeOH (20ml) was added W-2 Raney nickel (1g, dense slurry in EtOH) in MeOH (10ml). The mixture was cooled to 0°C and ammonia was bubbled through for 30min. The mixture was treated with H_2 (50psi) at room temperature for 65h. The catalyst was filtered off through Celite. The catalyst and Celite pad were washed with EtOH (300ml). Evaporation of the solvent from the combined filtrate and washings, followed by kugelrohr distillation, gave N,N′-bis(3-aminopropyl)propane-1,3-diamine (1.40g, 67%) as a colourless liquid: $bp_{0.08}$ 185°C, (lit. [Israel *et al*,1964] $bp_{0.07}$ 97-100°C); δ_H (270MHz; $[^2H]_6$-DMSO) 1.40-1.53 (6H, m, CH_2CH_2N), 1.67 (6H, br s, NH and NH_2), 2.47-2.56 (12H, m, CH_2N); *m/z* (FAB+) 189 [$(M+H)^+$, 100%].

N,N′-Bis(3-formamidopropyl)-N,N′-diformylpropane-1,3-diamine (109)

To N,N′-bis(3-aminopropyl)propane-1,3-diamine (2.00g, 10.6mmol) was added ethyl formate (50ml, 0.6mol) at room temperature. The solution was stirred at room temperature for 24h. Evaporation and chromatography ($MeOH/CH_2Cl_2$ 2:1) gave the title compound (2.21g, 85%) as a colourless oil: Found: C, 49.0; H, 8.19; N, 17.2. calc. for $C_{13}H_{24}N_4$: C, 51.99; H, 8.05; N, 18.65%; δ_H (400MHz, $[^2H]_6$-DMSO) 1.55-1.73 (6H, m, CH_2CH_2N), 3.03-3.07 (4H, m, CH_2), 3.15-3.26 (8H, m, CH_2), 7.99-8.05 (6H, m, CHO, and NH); *m/z* (FAB+) 301.1880 $(M+H)^+$ ($C_{13}H_{25}N_4O_4$ requires 301.1876); 302.1910 $(M+H)^+$ ($^{13}C_1C_{12}H_{25}N_4O_4$ requires 302.1909).

N,N′-Bis(2-cyanoethyl)-N,N′-dimethylpropane-1,3-diamine (120)

To N,N′-dimethyl-1,3-diaminopropane (4.64g, 45.4mmol) in MeOH (10ml) was added acrylonitrile (6.9ml, 5.6g, 104mmol) slowly at 0°C with stirring. The mixture was allowed to reach room temperature and was stirred for 24h. Evaporation gave the title compound (9.46g,

quant.) as a colourless liquid. A sample for elemental analysis was distilled using kugelrohr apparatus: $bp_{0.1}$ 215°C, (lit. [Schlögl and Schlögl, 1964] $bp_{1.0}$ 155-160°C); found: C, 64.1; H, 9.69; N, 27.0. calc. for $C_{11}H_{20}N_4$: C, 63.43; H, 9.68; N, 26.90%; δ_H (400MHz, $CDCl_3$) 1.61 (2H, qn, J 7.0Hz, CH_2CH_2N), 2.26 (6H, s, CH_3), 2.44 (4H, t, J 7.0Hz, CH_2), 2.50 (4H, t, J 7.0Hz, CH_2) 2.68 (4H, t, J 6.7Hz, CH_2); δ_C (100MHz; $CDCl_3$) 16.11, 25.02, 52.72, 54.30 (CH_2), 41.62 (CH_3), 119.17 (CN); *m/z* (EI+) 209.1722 M^+ ($^{13}C_1C_{10}H_{20}N_4$ requires 209.1722); 208.1695 M^+ ($C_{11}H_{20}N_4$ requires 208.1688).

N,N′-Bis(3-aminopropyl)-N,N′-dimethylpropane-1,3-diamine (121)

Method A

To N,N′-bis(2-cyanoethyl)-N,N′-dimethylpropane-1,3-diamine (1.184g, 5.69mmol) was added saturated NaOH solution in 95% EtOH (10ml) and W-2 Raney nickel (500mg, dense slurry in EtOH). The mixture was treated with H_2 (50psi) at 35°C for 48h. The catalyst was filtered off through Celite. The Celite pad and catalyst were washed with EtOH (150ml). The combined organic fractions were concentrated to low volume (about 5ml) and water (10ml) was added. Aqueous NaOH (*ca.* 75%) was added until the amine began to oil out. The amine was extracted with $CHCl_3$ (3× 100ml). The pooled organic phases were dried with $MgSO_4$. Evaporation and kugelrohr distillation gave the title compound (440mg, 36%) as a colourless liquid: $bp_{0.08}$ 145°C, (lit. [Schlöegl and Schlöegl, 1964] $bp_{0.6}$ 92-94°C); δ_H (270MHz, $[^2H]_6$-DMSO) 1.36 (4H, s, NH_2), 1.59 (6H, qn, J 7.0Hz, CH_2CH_2N), 2.19 (6H, s, CH_3), 2.33 (4H, t, J 7.6Hz, CH_2N), 2.37 (4H, t, J 7.0Hz, CH_2N), 2.71 (4H, t, J 7.0Hz, CH_2N).

Method B (tetrahydrochloride)

To N,N′-bis(2-cyanoethyl)-N,N′-dimethylpropane-1,3-diamine (1.350g, 6.5mmol) in absolute EtOH (20ml) was added W-2 Raney nickel (1g, dense slurry in EtOH). The mixture was cooled to 0°C and ammonia was bubbled through for 20min. The mixture was treated with H_2 (50psi) at room temperature for 90h. The catalyst was filtered off through Celite. The Celite pad and catalyst were washed with EtOH (150ml). Evaporation of the solvent from the combined filtrate and washings gave a blue oil which was dissolved in 95% EtOH (30ml). H_2S (generated by the addition of concentrated hydrochloric acid to FeS) was bubbled through for 5min, then N_2 for 30min. The black precipitate was removed by filtration through Celite. Evaporation gave a pale brown oil. Excess concentrated hydrochloric acid was added then evaporated. The solid was washed with absolute EtOH (10ml) to yield the title compound as its tetrahydrochloride (2.4g, quant.) as a pale yellow solid: mp 184-188°C; δ_H (270MHz, D_2O) 2.15-2.32 (6H, m, CH_2CH_2N), 2.96 (6H, s, CH_3), 3.15 (4H, t, J 7.7Hz, CH_2N), 3.29-3.36 (8H, m, CH_2NCH_3); *m/z* (FAB+) 218.2420 $(M+H)^+$ ($^{13}C_1C_{10}H_{29}N_4$ requires 218.2426); 217.2420 $(M+H)^+$ ($C_{11}H_{29}N_4$ requires 217.2392).

N,N′-Bis(3-trifluoroacetamidopropyl)-N,N′-dimethylpropane-1,3-diamine (124)

To **121** (400mg, 1.85mmol) in MeOH (2ml) was added ethyl trifluoroacetate (1.5ml, 8.85mmol) at room temperature. The mixture was stirred for 8h. Evaporation and chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 40:10:0.2) gave the title compound (731mg, 97%) as a colourless oil: δ_H (400MHz; $[^2H]_6$-DMSO) 1.91 (6H, qn, J 7.0Hz, CH_2CH$_2$N), 2.57 (6H, s, CH_3), 2.82 (8H, br t, J 6.4Hz, CH_2NCH$_3$), 3.41 (4H, q, J 6.4Hz, CH_2NCOCF_3), 9.72 (2H, s, NH); *m/z* (FAB+) 409.2039 $(M+H)^+$ ($C_{15}H_{27}F_6N_4O_2$ requires 409.2038); 313 [$(M-F_3CCO+H)^+$, 100%]; 217 [$(M-2\times F_3CCO+H)^+$, 79%].

***t*-Butyl N-methyl-N-(3-methylaminopropyl)carbamate (126)**

To a solution of N,N′-dimethylpropane-1,3-diamine (1.53g, 15.0mmol) in dry THF (10ml) at 0°C was added di-*t*-butyl dicarbonate (1.10g, 5.0mmol) in dry THF (10ml) dropwise over 40 min with stirring. When addition was complete, the mixture was stirred at 0°C for a further 1h, then at room temperature for 20h. Evaporation and chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 30:10:0.5) gave the title compound (723mg, 71%) as a colourless liquid: δ_H (270MHz; $CDCl_3$) 1.46 (9H, s, Bu^t), 1.69-1.82 (2H, m, CH_2CH$_2$N), 2.46 (3H, s, NCH_3), 2.61 (2H, t, J 7.0Hz, CH_2N), 2.85 (3H, s, CH_3NBoc), 3.17 (1H, br s, NH), 3.26-3.35 (2H, m, CH_2NBoc); *m/z* (FAB+) 204.1795 $(M+H)^+$ ($^{13}C_1C_9H_{23}N_2O_2$ requires 204.1793); 203.1763 $(M+H)^+$ ($C_{10}H_{23}N_2O_2$ requires 203.1760).

N,N′-Bis(3-(N-*t*-butoxycarbonyl-N-methylamino)propyl)-N,N′-dimethylpropane-1,3-diamine (127)

To **126** (277mg, 1.37mmol) in dry DMF (4ml) was added anhydrous K_2CO_3 (200mg, 1.45mmol) and 1,3-dibromopropane (138mg, 0.68mmol). The mixture was heated at 80°C for 16h, then the DMF was evaporated. The residue was extracted with CH_2Cl_2 (4× 30ml). Evaporation and chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 70:10:1) gave the title compound (160mg, 52%) as a pale yellow oil: δ_H (270MHz, $CDCl_3$) 1.45 (18H, s, Bu^t), 1.63-1.71 (6H, m, CH_2CH$_2$N), 2.21 (6H, s, NCH_3), 2.30-2.37 (8H, m, CH_2NCH$_3$), 2.85 (6H, s, BocNCH_3), 3.22 (4H, t, CH_2NBoc, J 7.0Hz).

This material was used immediately without further characterisation.

N,N′-Bis(3-methylaminopropyl)-N,N′-dimethylpropane-1,3-diamine tetrahydrochloride (110)

To **127** (115mg, 0.26mmol) was added 5M hydrochloric acid (5ml) and the mixture was stirred overnight at room temperature. Evaporation gave a pale brown solid which was washed with CH_2Cl_2 (20ml) to give the title compound (100mg, quant.) as an off white solid: mp 217-220°C; δ_H (270MHz; D_2O) 2.15-2.30 (6H, m, CH_2CH$_2$N), 2.75 (6H, s, CH_3), 2.94 (6H, s, CH_3), 3.15

(4H, t, J 7.9Hz, CH_2), 3.27-3.35 (8H, m, CH_2); *m/z* (FAB+) 317 [(M+3H + 2× $^{35}Cl^-$)$^+$, 9%], 281 [(M+2H + $^{35}Cl^-$)$^+$, 45%], 246.2729 (M+H)$^+$ ($^{13}C_1C_{12}H_{33}N_4$ requires 246.2739), 245.2709 (M+H)$^+$ ($C_{13}H_{33}N_4$ requires 245.2705).

N,N′-Bis(2-hydroxy-3-(ω-methoxyPEG 550)propyl-(3-methylaminopropyl))-N,N′-dimethylpropane-1,3-diamine tetrahydrochloride (150)

To **110** (83mg, 0.21mmol) in *iso*propanol (1ml) was added 5M aqueous NaOH (213µl, 1.1mmol) and the mixture was stirred for 5min at room temperature. mPEG 550 oxiranylmethyl ether (255mg, 0.4mmol) in *iso*propanol (1ml) was added and the mixture was heated to 75°C for 24h. Evaporation gave a pale brown residue. The product was extracted with CH_2Cl_2 (3× 10ml). Evaporation then chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 50:10:1 then, 40:10:1) gave a colourless oil (98mg, 32%). The sample was converted to its tetrahydrochloride salt by the addition of 5M hydrochloric acid (2ml). The acid was evaporated then the sample was lyophilised to yield an off-white wax (105mg): δ_H (free amine, 400MHz; $CDCl_3$) 1.80-1.90 (6H, m, C*H*$_2$CH$_2$N), 2.40-2.49 (12H, m, CH_3), 2.57-2.70 (16H, m, CH_2N), 3.38 (6H, s, OCH_3), 3.49-3.51 (4H, m, CH_2O), 3.54-3.56 (4H, m, CH_2O), 3.64-3.66 (*Ca.* 96H, m, CH_2O), 3.97-4.03 (2H, m, C*H*OH) *m/z* (FAB+) $^{12}C/^{13}C$ peak cluster centred at: 1390 (7%), 1346 (5%), 1302 (7%), 1258 (7%), 1214 (5%), 1170 (6%), 1126 (4%), all (M+H)$^+$.

N,N′-Di-(*t*-butoxycarbonyl)-N,N′-bis(2-cyanoethyl)propane-1,3-diamine (129)

To N,N′-bis(2-cyanoethyl)propane-1,3-diamine (2.50g, 13.9mmol) in CH_2Cl_2 (25ml) was added di-*t*-butyl dicarbonate (6.06g, 27.8mmol) in CH_2Cl_2 (10ml) slowly in 10 portions at 0°C. After 15min, the stirred solution was allowed to reach room temperature and was stirred for a further 5h. Evaporation gave the title compound (5.3g, quant.) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.48 (18H, s, Bu^t), 1.81 (2H, qn, J 7.3Hz, C*H*$_2$CH$_2$N), 2.62 (4H, m, CH_2CN), 3.29 (4H, t, J 7.3Hz, CH_2NBoc), 3.48 (4H, t, J 6.5Hz, NCCH$_2$C*H*$_2$NBoc).

This material was used immediately without further characterisation.

N,N′-Bis(3-aminopropyl)-N,N′-di(-*t*-butoxycarbonyl)propane-1,3-diamine (130)

Method A

Lithium aluminium hydride (2.60g, 69mmol) was suspended in dry Et_2O (200ml) under N_2. The suspension was added to **129** (3.58g, 9.4mmol) in dry THF (40ml) dropwise at 0°C under N_2. The mixture was stirred for 2h at 0°C under N_2. Excess hydride was quenched by the dropwise addition of aqueous 1M NaOH to yield a white precipitate. The solvents were decanted and the residue was extracted with Et_2O (3× 50ml). Evaporation of the pooled organic phases, then chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 20:10:1) gave the title compound (790mg, 22%) as a colourless oil: δ_H (400MHz, [^{2}H]$_6$-DMSO) 1.39 (18H, s, Bu^t),

1.48-1.68 (10H, m, C*H*$_2$CH$_2$N and NH$_2$), 2.50 (4H, t, CH$_2$N, 7.0Hz), 3.10 (4H, t, CH$_2$NBoc, J 7.0Hz), 3.17 (4H, t, CH$_2$NBoc, J 6.8Hz) *m/z* (FAB+) 389 [(M+H)$^+$ 39%], 189 [(M− 2× Boc +H)$^+$ 47%]; and a by-product, N-(3-aminopropyl)-N,N′-di(-*t*-butoxycarbonyl)propane-1,3-diamine **133**, (1.15g, 37%) as a colourless oil: δ_H (270MHz, CDCl$_3$) 1.44 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.67 (4H, m, C*H*$_2$CH$_2$N), 2.74 (2H, m, C*H*$_2$NH$_2$), 3.13-3.35 (8H, m, CH$_2$NBoc and NH$_2$), 3.67 (1H, m, NH); *m/z* (FAB+) 332 [(M+H)$^+$, 100%], 232 [(M−Boc +H)$^+$, 20%], 132 [(M−2× Boc+H)$^+$, 28%].

Method B

To **129** (2.74g, 7.2mmol) in MeOH (20ml) was added W-2 Raney nickel (1g, dense slurry in water) with MeOH (10ml). The mixture was cooled to 0°C and ammonia was bubbled through for 30min. The mixture was treated with H_2 (50psi) for 72h. The catalyst was filtered off through Celite. The Celite pad and catalyst were washed with EtOH (400ml). Evaporation of the solvent from the combined filtrate and washings, followed by chromatography (CH$_2$Cl$_2$/MeOH/conc. aqueous NH$_3$ 20:10:1) gave the title compound (1.93g, 69%) as a colourless oil with properties as above.

Method C

To **129** (1.120g, 1.93mmol) in MeOH (10ml) was added concentrated aqueous ammonia (8ml). Ammonia was bubbled through the mixture for 10 min at 0°C. The mixture was heated in a sealed vessel, with stirring, at 60°C for 7h, then at room temperature overnight. Evaporation and chromatography (CH$_2$Cl$_2$/MeOH/conc. aqueous NH$_3$ 20:10:1) gave the title compound (418mg, 56%) as a colourless oil with properties as above.

N,N′-Bis(3-trifluoroacetamidopropyl)propane-1,3-diamine (134)

To thermine (5.1g, 27mmol) in MeOH (10ml) was added ethyl trifluoroacetate (7.9g, 56mmol) slowly at 0°C. After 5min the ice bath was removed and stirring was continued for 150min. Evaporation gave the title compound (10.4g, quant.) as a colourless oil: δ_H (400MHz, CDCl$_3$) 1.67 (2H, qn, J 7.0Hz, C*H*$_2$CH$_2$N), 1.73 (4H, qn, J 6.1Hz, C*H*$_2$CH$_2$NCO), 2.66 (4H, t, J 7.0Hz, CH$_2$N), 2.79 (4H, t, J 5.9Hz, CH$_2$N), 3.44 (4H, t, J 6.1Hz, CH$_2$NCO), 6.8-10.2 (4H, br signal, NH); δ_C (100MHz, CDCl$_3$) 27.12 (CH$_2$), 30.03 (CH$_2$), 40.23 (CH$_2$), 48.02 (CH$_2$), 48.77 (CH$_2$), 116.2 (q, J 288Hz, CF$_3$), 157.2 (q, J 37Hz, *C*OCF$_3$).

This material was used immediately without further characterisation.

N,N′-Bis(3-trifluoroacetamidopropyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (131)

Method A

To **134** (180mg, 0.47mmol) in dry THF (2ml) was added di-*t*-butyl dicarbonate (215mg, 0.99mmol) in dry THF (2ml) dropwise at 0°C. After 5min, the ice bath was removed and the mixture was stirred at room temperature overnight. Evaporation and chromatography (EtOAc/hexane 1:1) gave the title compound (254mg, 93%) as a white solid. A sample for elemental analysis was recrystallised from EtOAc/hexane: mp 70-71°C; Found: C, 47.7; H, 6.7; N, 9.6. calc. for $C_{23}H_{38}N_4O_6F_6$: C, 47.58; H, 6.60; N, 9.65%; δ_H (400MHz, $CDCl_3$) 1.47 (18H, s, Bu^t), 1.74-1.80 (6H, m, CH_2CH$_2$N), 3.11-3.19 (4H, m, CH$_2$NCOCF$_3$), 3.21-3.40 (8H, m, CH$_2$NBoc), 8.29 (2H, br s, NH); δ_C (100MHz, $CDCl_3$) 27.14 (CH_2), 27.67 (CH_2), 28.37 (CH_3), 35.95 (CH_2), 43.11 (CH_2), 44.86 (CH_2), 80.61 (Boc C), 116.0 (q, J 287Hz, CF_3), 156.8 (q, J 37Hz, *C*OCF$_3$); *m/z* (FAB–) 579 [(M–H)$^-$, 100%]; (FAB+) 603 [(M+Na)$^+$, 38%], 581 [(M+H)$^+$, 26%], 481 [(M–Boc +H)$^+$, 52%], 381 [(M–2× Boc +H)$^+$, 14%].

Method B

To **130** (620mg, 1.60mmol) in MeOH (10ml) was added ethyl trifluoroacetate (1ml, 8mmol). The mixture was stirred at room temperature overnight. Evaporation and chromatography (EtOAc/hexane 1:1) gave the title compound (890mg, 96%) as a white solid with properties as above.

N,N′-Bis(3-(N-methyltrifluoroacetamido)propyl)-N,N′-di-(*t*-butoxycarbonyl-)propane-1,3-diamine (132)

To **131** (712mg, 1.23mmol) in dry THF (20ml) was added potassium *t*-butoxide (2.6ml, 1.0M solution in THF, 2.6mmol), then further dry THF (5ml) at room temperature under N_2. After 5min, methyl iodide (0.2ml, 456mg, 3mmol) was added and the mixture was stirred at room temperature for 20min, under N_2. The mixture was concentrated to low volume, diluted with EtOAc (200ml), washed with water (200ml), then brine (200ml) and then dried with $MgSO_4$. Evaporation gave the title compound (730mg, 98%) as a colourless oil: δ_F (376MHz, [^{2}H]$_6$-DMSO) –68.17 (2F, s), –69.11 (4F, s); δ_H (400MHz, [^{2}H]$_6$-DMSO, 22°C) 1.37 (18H, s, Bu^t), 1.65-1.77 (6H, m, CH_2CH$_2$N), 2.95 (2H, s, CH$_3$N), 3.09-3.15 (11H, m, CH$_2$NCO and CH$_3$N), 3.32-3.44 (5H, m, CH$_2$NCO); δ_H (400MHz, [^{2}H]$_6$-DMSO, 100°C) 1.42 (18H, s, Bu^t), 1.68-1.80 (6H, m, CH_2CH$_2$N), 2.96 (6H, s, CH$_3$N), 3.10-3.20 (8H, m, CH$_2$NBoc), 3.41 (4H, t, J 7.6Hz, CH$_2$NCOCF$_3$); *m/z* (FAB+) 610.3122 (M+H)$^+$ (^{13}C$_1$C$_{24}$H$_{43}$F$_6$N$_4$O$_6$ requires 610.3120), 609.3091 (M+H)$^+$ ($C_{25}H_{43}F_6N_4O_6$ requires 609.3087).

N,N′-Bis(3-methylaminopropyl)-N,N′-di(*t*-butoxycarbonyl)propane-1,3-diamine (128)

To **132** (144mg, 0.24mmol) in MeOH (8ml) was added concentrated aqueous ammonia solution (2ml). The mixture was stirred at 55°C in a sealed bottle for 3½h. Evaporation and chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 40:20:2.5) gave the title compound (78mg, 79%) as a pale yellow oil: δ_H (400MHz, $CDCl_3$) 1.45 (18H, s, Bu^t), 1.72-1.74 (6H, m, C*H*$_2$CH$_2$N), 2.39 (6H, s, CH_3N), 2.58 (4H, t, J 7.0Hz, CH_2NHMe), 2.76 (2H, s, NH), 3.12-3.20 (4H, m, CH_2NBoc), 3.22-3.28 (4H, m, CH_2NBoc); *m/z* (FAB+) 418.3473 $(M+H)^+$ ($^{13}C_1C_{20}H_{45}N_4O_4$ requires 418.3474), 417.3443 $(M+H)^+$ ($C_{21}H_{45}N_4O_4$ requires 417.3441), 317 [(M–Boc +H)$^+$, 15%], 217 [(M– 2× Boc +H$^+$), 38%].

N,N′-Bis(3-methylaminopropyl)propane-1,3-diamine tetrahydrochloride (159)

HCl was bubbled through a solution of **128** (75mg, 0.18mmol) in CH_2Cl_2 (10ml) for 10min. Evaporation and lyophilisation gave the title compound (61mg, 96%) as a hygroscopic white solid: δ_H (400MHz, D_2O) 2.08-2.20 (6H, m, C*H*$_2$CH$_2$N), 2.75 (6H, s, CH_3), 3.12-3.24 (12H, m, CH_2N); *m/z* (FAB+) 217 [(M+H)$^+$, 76%].

(±)-N,N′-Bis(3-(N-(2-hydroxy-3-phenoxypropyl)-N-methylamino)propyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (136)

To **128** (45mg, 0.11mmol) in *iso*propanol (1ml) was added phenoxymethyloxirane (33mg, 0.22mmol) and the mixture was heated to reflux with stirring for 10h. Evaporation and preparative TLC (CH_2Cl_2/MeOH/conc. aqueous NH_3 70:10:0.5) gave the title compound (20mg, 26%) as a colourless oil: δ_H (270MHz, $CDCl_3$) 1.45 (18H, s, Bu^t), 1.65-1.80 (6H, m, C*H*$_2$CH$_2$N), 2.32 (6H, s, CH_3N), 2.44-2.60 (8H, m, CH_2N), 3.10-3.35 (10H, m, CH_2NBoc and OH), 3.95-3.99 (4H, m, CH_2O), 4.05-4.11 (2H, m, C*H*OH), 6.90-6.98 (6H, m, ArH), 7.25-7.31 (4H, m, ArH); *m/z* (FAB+) 718.4853 $(M+H)^+$ ($^{13}C_1C_{38}H_{65}N_4O_8$ requires 718.4836); 717.4811 $(M+H)^+$ ($C_{39}H_{65}N_4O_8$ requires 717.4802).

N,N′-Bis-(3-(N-(2-hydroxy-3-(ω-methoxyPEG 2000)propyl)-N-methylamino)propyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (137)

To **128** (43mg, 0.103mmol) in *iso*propanol (1ml) was added mPEG 2000 oxiranylmethyl ether (410mg, 0.21mmol) in *iso*propanol (2ml) and the mixture was heated to reflux with stirring for 27h. Evaporation gave the title compound (450mg, quant.) as a pale yellow wax: δ_H (400MHz, $CDCl_3$) 1.45 (18H, s, Bu^t), 1.66-1.75 (6H, m, C*H*$_2$CH$_2$N), 2.25 (6H, s, CH_3N), 2.31-2.45 (8H, m, CH_2N), 3.15-3.20 (8H, m, CH_2NBoc), 3.38 (6H, s, OCH_3), 3.42-3.60 (*ca.* 12H, CH_2O), 3.60-3.77 (*ca.* 360H, CH_2O), 3.81-3.88 (*ca.* 4H, m, CH_2O and C*H*OH).

N,N′-Bis(3-(N-(2-hydroxy-3-(ω-methoxyPEG 2000)propyl)-N-methylamino)propyl)propane-1,3-diamine tetrahydrochloride (152)

HCl was bubbled through a stirred solution of **137** (100mg, 0.02mmol) in CH_2Cl_2 (10ml) for 10min. Evaporation and lyophilisation gave the title compound (100mg, quant.) as a white wax: δ_H (400MHz, $CDCl_3$) 2.43 (6H, m, C*H*$_2$CH$_2$N), 2.98 (6H, s, CH_3N), 3.31-3.36 (16H, m, CH_2N), 3.38 (6H, s, CH_3O), 3.48-3.83 (*ca.* 370H, m, CH_2O), 4.33 (2H, m, C*H*OH).

ω-Methoxy PEG 550 oxiranylmethyl ether

To mPEG 550 (10.0g, 0.018mol, previously dried by azeotropic removal of water with toluene) was added NaOH (2.18g, 0.054mol), water (0.3ml) and epichlorohydrin (17ml, 0.22mol). The mixture was stirred vigorously at 60°C for 3h. The mixture was diluted with CH_2Cl_2 (100ml) and the solid was filtered off and washed with CH_2Cl_2 (250ml). The combined organic fractions were dried with $MgSO_4$. Evaporation under high vacuum for 6h gave the title compound (10.8g, 98%) as a colourless oil: δ_H (270MHz, [^{2}H]$_6$-DMSO) 2.53 (1H, dd, J 5.1, 2.5Hz, oxirane CH_2), 2.72 (1H, t, J 5.0Hz, oxirane CH_2), 3.06-3.12 (1H, m, oxirane CH), 3.22-3.31 (2H, m, CHC*H*$_2$O), 3.24 (3H, s, CH_3O), 3.41-3.73 (*ca.* 45H, m, CH_2O); *m/z* (ES+) 771 (20%), 727 (36%), 683 (56%), 639 (74%), 595 (92%), 551 (100%), 507 (92%), 463 (80%), 419 (58%), 375 (46%) all $(M+Na)^+$.

N,N′-Bis(3-(N-(2-hydroxy-3-(ω-methoxyPEG 550)propyl)-N-methylamino)propyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine

To **128** (36mg, 0.086mmol) in *iso*propanol (2ml) was added mPEG 550 oxiranylmethyl ether (104mg, 0.17mmol) and the mixture was heated to reflux with stirring for 24h. Further amine (15mg) in *iso*propanol (2ml) was added and the mixture was heated to reflux with stirring for a further 5h. Evaporation gave the title compound (143mg, quant.) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.45 (18H, s, Bu^t), 1.61-1.69 (6H, m, C*H*$_2$CH$_2$N), 2.25 (6H, s, CH_3N), 2.29-2.46 (8H, m, CH_2N), 3.17-3.21 (8H, m, CH_2NBoc), 3.38 (6H, s, CH_3O), 3.42-3.61 (*ca.* 10H, CH_2O) 3.63-3.66 (*ca.* 90H, CH_2O), 3.83-3.86 (2H, m, C*H*OH); *m/z* (FAB+) $^{12}C/^{13}C$ peak cluster centred at: 1474 (1%), 1430 (1%), 1386 (1%), 1342 (1%) all $(M+H)^+$.

N,N′-Bis(3-(N-(2-hydroxy-3-(ω-methoxyPEG 550)propyl)-N-methylamino) propyl)propane-1,3-diamine tetrahydrochloride (151)

HCl was bubbled through a stirred solution of the Boc-protected diPEG 550 conjugate (90mg, 0.06mmol) in CH_2Cl_2 (3ml) for 10min. Evaporation and lyophilisation gave the title compound (90mg, quant.) as a off white semi-solid: δ_H (400MHz, $CDCl_3$) 2.47 (6H, m, C*H*$_2$CH$_2$N), 3.02 (6H, br s, CH_3N), 3.30-3.45 (16H, m, CH_2N), 3.38 (6H, s, CH_3O), 3.47-3.82 (*ca.* 100H,

CH_2O), 4.33 (2H, m, C*H*OH); *m/z* (FAB+) 1406 (2%), 1362 (2.5%), 1318 (3%), 1274 (3%), 1230 (2.5%), 1186 (2.5%), 1142 (2%) all $(M+H)^+$.

N,N′-Bis(3-(N-(2-hydroxy-3-(ω-methoxyPEG 550)propyl)amino)propyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (139)

preliminary experiment

To **130** (82mg, 0.21mmol) in *iso*propanol (2ml) was added mPEG 550 oxiranylmethyl ether (253mg, 0.42mmol) in *iso*propanol (1ml). The mixture was heated to reflux, with stirring, for 24h. Evaporation gave the crude diPEGylated conjugate (330mg, quant.) as a pale yellow oil: δ_H (400MHz, $CDCl_3$), crude, 1.41 (18H, s, Bu^t), 1.62-1.95 (6H, m, C*H*$_2$$CH_2N$), 2.51-2.59 (8H, m, CH_2N), 3.15-3.25 (8H, m, CH_2NBoc), 3.35 (6H, s, CH_3O), 3.41-3.53 (*ca.* 12H, CH_2O), 3.60-3.78 (*ca.* 106H, CH_2O), 3.80-3.83 (4H, m, CH_2O and C*H*OH); *m/z* (FAB+) $^{12}C/^{13}C$ peak cluster centred at: 1490 (3%), 1446 (3%), 1402 (3%), 1358 (3%), 1314 (3%) all $(M+H)^+$.

N,N,N′,N′-Tetrakis(3-(N-2-hydroxy-3-(ω-methoxyPEG550)propyl)amino)propyl-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (141) and N,N,N′-Tris(3-(N-2-hydroxy-3-(ω-methoxyPEG550)propyl)amino)propyl-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (140)

To **130** (1.75g, 4.51mmol) in *iso*propanol (20ml) was added mPEG 550 oxiranylmethyl ether (5.4g, 9mmol) in *iso*propanol (20ml). The mixture was heated, with stirring, at 80°C for 38h. Evaporation and chromatography (CH_2Cl_2/MeOH 6:1) gave the tetraPEGylated conjugate (1.40g, 11%) as a pale yellow oil: δ_H (400MHz, $CDCl_3$): 1.45 (18H, s, Bu^t), 1.60-1.70 (6H, m, C*H*$_2$$CH_2N$), 2.50-2.55 (12H, m, CH_2N), 3.15-3.26 (8H, m, CH_2NBoc), 3.38 (12H, s, CH_3O), 3.41-3.49 (*ca.* 6H, m, CH_2O), 3.51-3.56 (*ca.* 8H, m, CH_2O), 3.56-3.68 (*ca.* 180H, m, CH_2O), 3.80-3.84 (4H, CH_2O and C*H*OH); *m/z* (ES+) $^{12}C/^{13}C$ peak cluster centred at: 1339 (82%), 1318 (95%), 1296 (90%), 1274 (100%), 1252 (95%), 1230 (90%), 1208 (80%) all $(M+H)^{2+}$; and the triPEGylated conjugate (560mg, 24%) as a pale yellow oil: 1.47 (18H, s, Bu^t), 1.60-1.70 (4H, m, C*H*$_2$$CH_2N$), 1.95-2.05 (2H, m, C*H*$_2$$CH_2N$), 2.48-2.60 (6H, m, CH_2N), 2.95-3.05 (4H, m, CH_2N), 3.15-3.25 (8H, m, CH_2NBoc), 3.41 (9H, s, CH_3O), 3.44-3.50 (*ca.* 4H, m, CH_2O), 3.54-3.58 (*ca.* 6H, m, CH_2O), 3.58-3.80 (*ca.* 130H, m, CH_2O), 3.80-3.94 (3H, m, C*H*OH) *m/z* (ES+) $^{12}C/^{13}C$ peak cluster centred at: 1098 (85%), 1076 (90%), 1054 (100%), 1031 (96%), 1009 (100%), 988 (98%), 966 (90%) all $(M+H)^{2+}$.

N,N,N′-Tris(3-(N-2-hydroxy-3-(ω-methoxyPEG550)propyl)amino)propyl-propane-1,3-diamine tetrahydrochloride (153)

HCl was bubbled through a stirred solution of **140** (2.27g, 1.1mmol) in CH_2Cl_2 (40ml) for 30min. Evaporation and lyophilisation gave the title compound (2.07g, quant.) as an off white foam: δ_H (400MHz, D_2O) 2.13 (6H, m, C*H*$_2$CH$_2$N), 3.11-3.27 (18H, m, CH_2N), 3.39 (9H, s, CH_3O), 3.53-3.64 (*ca.* 16H, m, CH_2O), 3.68-3.88 (*ca.* 120H, m, CH_2O), 4.14 (3H, m, C*H*OH).

N,N,N′,N′-Tetrakis(3-(N-2-hydroxy-3-(ω-methoxyPEG550)propyl)amino)propyl-propane-1,3-diamine tetrahydrochloride (154)

HCl was bubbled through a stirred solution of **141** (1.34g, 0.52mmol) in CH_2Cl_2 (25ml) for 30min. Evaporation and lyophilisation gave the title compound (1.30g, quant.) as an off white foam: δ_H (400MHz, D_2O) 2.10 (6H, m, C*H*$_2$CH$_2$N), 2.97-3.22 (20H, m, CH_2N), 3.39 (12H, s, CH_3O), 3.53-3.64 (*ca.* 20H, m, CH_2O), 3.66-3.72 (*ca.* 164H, m, CH_2O), 4.17 (4H, m, C*H*OH).

N-(3-Aminopropyl)-N′-(3-*t*-butoxycarbonylaminopropyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (145)

Method A

To thermine (2.09g, 11.1mmol) in absolute EtOH (50ml) was added ethyl trifluoroacetate (1.74g, 12.3mmol) in absolute EtOH (50ml) dropwise over 40min, with stirring, at −78°C. The mixture was then stirred for a further 30min at 0°C. Di-*t*-butyl dicarbonate (9.7g, 44.5mmol) in absolute EtOH (20ml) was added dropwise over 10min, stirring was continued at 0°C for 30min then at room temperature overnight. Evaporation gave a yellow oil which was dissolved in MeOH (100ml), to which concentrated aqueous ammonia solution was added (70ml), then ammonia gas was bubbled through the solution for 30min, with stirring, at 0°C. The mixture was stirred in a sealed bottle for 20h. Evaporation then chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 70:10:1, then 50:10:1, then 30:10:1) gave the title compound (2.76g, 51%) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.44 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.47 (9H, s, Bu^t), 1.60-1.67 (2H, m, C*H*$_2$CH$_2$N), 1.73-1.78 (4H, m, C*H*$_2$CH$_2$N), 1.97 (2H, s, NH_2), 2.71 (2H, t, J 6.7Hz, CH_2NH_2), 3.10-3.28 (10H, m, CH_2NBoc), 4.85 (0.5H, br s, NH), 5.30 (0.5H, br s, NH); *m/z* (FAB+) 490.3678 $(M+H)^+$ ($^{13}C_1C_{23}H_{49}N_4O_6$ requires 490.3686); 489.3651 $(M+H)^+$ ($C_{24}H_{49}N_4O_6$ requires 489.3652).

Method B

To thermine (1.118g, 5.95mmol) in THF (100ml) was added ethyl trifluoroacetate (844mg, 5.95mmol) in THF (20ml) slowly over 5min, with stirring, at 0°C. The mixture was stirred for 10min at 0°C then di-*t*-butyl dicarbonate (5.2g, 23.9mmol) in THF (25ml) was added slowly over 40min at 0°C. The mixture was allowed to reach room temperature and stirred for 2h.

Evaporation gave a colourless oil which was dissolved in propylamine (50ml) and heated to reflux, with stirring, for 16h. Evaporation and chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 70:10:1, then 50:10:1, then 30:10:1) gave the title compound (1.184g, 40%) as a colourless oil with spectroscopic properties as above.

N-(3-(N-(2-Hydroxy-3-(ω-methoxyPEG 550)propyl)amino)propyl)-N′-(3-*t*-butoxy carbonylaminopropyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (146) and N,N-Bis(3-(N-(2-hydroxy-3-(ω-methoxyPEG 550)propyl)amino)propyl)-N′-(3-*t*-butoxycarbonylaminopropyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (148)

To **145** (959mg, 1.97mmol) in *iso*propanol (5ml) was added mPEG 550 oxiranylmethyl ether (1.19g, 1.97mmol) in *iso*propanol (5ml). The mixture was heated to reflux, with stirring, for 48h. Evaporation and chromatography (CH_2Cl_2/MeOH 7:1) gave the diPEGylated conjugate (315mg, 9%) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.44 (9H, s, Bu^t), 1.45 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.62-1.74 (6H, m, C*H*$_2$CH$_2$N), 2.48-2.60 (6H, m, CH$_2$N), 3.15-3.24 (10H, m, CH$_2$NBoc), 3.38 (6H, s, CH$_3$O), 3.50-3.57 (*ca.* 12H, m, CH$_2$O), 3.60-3.73 (*ca.* 90H, m, CH$_2$O), 3.81-3.85 (2H, m, C*H*OH); *m/z* (FAB+) 1590 (16%), 1546 (21%), 1501 (21%), 1457 (20%), 1413 (20%), 1369 (19%) all $(M+H)^+$; and the monoPEGylated conjugate (460mg, 21%) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.44 (9H, s, Bu^t), 1.45 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.65-1.78 (6H, m, C*H*$_2$CH$_2$N), 2.90-2.98 (4H, m, CH$_2$N), 3.14-3.27 (10H, m, CH$_2$NBoc), 3.38 (3H, s, CH$_3$O), 3.54-3.58 (*ca.* 6H, m, CH$_2$O), 3.62-3.66 (*ca.* 44H, m, CH$_2$O), 3.80-3.84 (1H, m, C*H*OH); *m/z* (FAB+) 1149 (10%), 1105 (18%), 1061 (20%), 1017 (25%), 973 (24%), 929 (20%) all $(M+H)^+$; (FAB+) 1061.7005 $(M+H)^+$ ($C_{50}H_{101}N_4O_{19}$ requires 1061.7060), 1017.6772 $(M+H)^+$ ($C_{48}H_{97}N_4O_{18}$ requires 1017.6798), 973.6511 $(M+H)^+$ ($C_{46}H_{93}N_4O_{17}$ requires 973.6536).

N,N-Bis(3-(N-(2-hydroxy-3-(ω-methoxyPEG 550)propyl)amino)propyl)-N′-(3-*t*-butoxycarbonylaminopropyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (148)

To **145** (188mg, 0.39mmol) in *iso*propanol (1ml) was added mPEG 550 oxiranylmethyl ether (462mg, 0.77mmol) in *iso*propanol (1ml). The mixture was heated at 75°C, with stirring, for 24h. Evaporation and chromatography (CH_2Cl_2/MeOH 8:1 then 7:1) gave the title compound (398mg, 61%) as a colourless oil with spectroscopic properties as above.

N-(3-(N-(2-Hydroxy-3-(ω-methoxyPEG 550)propyl)amino)propyl)-N′-(3-aminopropyl)propane-1,3-diamine tetrahydrochloride (155)

HCl was bubbled through a stirred solution of **146** (372mg, 0.34mmol) in CH_2CH_2 (30ml) for 20min at room temperature. Evaporation and lyophilisation gave the title compound (330mg, quant.) as a white wax: δ_H (270MHz, D_2O) 2.12 (6H, m, C*H*$_2$CH$_2$N), 3.10-3.20 (14H, m, CH_2N), 3.38 (3H, s, CH_3O), 3.64-3.72 (*ca.* 45H, CH_2O), 4.05 (1H, m, C*H*OH); *m/z* (FAB+) 849 (45%), 805 (55%), 761 (64%), 717 (60%), 673 (50%), 630 (40%) all $(M+H)^+$.

N,N-Bis(3-(N-(2-hydroxy-3-(ω-methoxyPEG 550)propyl)amino)propyl)-N′-(3-aminopropyl)propane-1,3-diamine tetrahydrochloride (157)

HCl was bubbled through a stirred solution of **148** (450mg, 0.28mmol) in CH_2CH_2 (10ml) for 20min at room temperature. Evaporation and lyophilisation gave the title compound (408mg, quant.) as a pale yellow wax: δ_H (400MHz, D_2O) 2.14-2.23 (6H, m, C*H*$_2$CH$_2$N), 3.10-3.25 (10H, m, CH_2N), 3.35-3.46 (12H, s, CH_2N and CH_3O), 3.62-3.85 (*ca.* 100H, CH_2O), 4.23 (2H, m, C*H*OH); *m/z* (FAB+) $^{12}C/^{13}C$ peak cluster centred at 1334 (3%), 1290 (4%), 1246 (4%), 1202 (5%), 1158 (5%), 1114 (3.5%), 1070 (3.5%) all $(M+H)^+$.

N-(3-(N-(2-Hydroxy-3-(ω-methoxyPEG 2000)propyl)amino)propyl)-N′-(3-*t*-butoxycarbonylaminopropyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (147) and

N,N-Bis(3-[N-(2-hydroxy-3-(ω-methoxyPEG 2000)propyl)amino)propyl)-N′-(3-*t*-butoxycarbonylaminopropyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (149)

To **145** (986mg, 2.02mmol) in *iso*propanol (20ml) was added mPEG 2000 oxiranylmethyl ether (4.15g, 2.02mmol) in *iso*propanol (1ml). The mixture was heated to reflux, with stirring, for 100h. Evaporation and chromatography (CH_2Cl_2/MeOH 8:1 and then 7:1) gave the diPEGylated conjugate (2.53g, 28%) as a pale yellow wax: δ_H (400MHz, $CDCl_3$) 1.44 (9H, s, Bu^t), 1.45 (9H, s, Bu^t), 1.47 (9H, s, Bu^t), 1.65-1.75 (6H, m, C*H*$_2$CH$_2$N), 2.48-2.59 (6H, m, CH_2N), 3.08-3.26 (10H, m, CH_2NBoc), 3.39 (6H, s, CH_3O), 3.45-3.49 (*ca.* 8H, m, CH_2O), 3.55-3.72 (*ca.* 360H, m, CH_2O), 3.82 (2H, m, C*H*OH); and the monoPEGylated conjugate (1.80g, 35%) as a pale yellow wax: δ_H (400MHz, $CDCl_3$) 1.44 (9H, s, Bu^t), 1.45 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.66-1.75 (6H, m, C*H*$_2$CH$_2$N), 2.85-2.91 (4H, m, CH_2N), 3.10-3.25 (10H, m, CH_2NBoc), 3.38 (3H, s, CH_3O), 3.54-3.56 (*ca.* 8H, m, CH_2O), 3.64-3.70 (*ca.* 186H, m, CH_2O), 3.82 (1H, m, C*H*OH); *m/z* (FAB+) $^{12}C/^{13}C$ peak cluster centred at 2416 (1%), 2374 (2%), 2324 (5%), 2288 (5%), 2239 (4%) all $(M+H)^+$.

N-(3-(N-(2-Hydroxy-3-(ω-methoxyPEG 2000)propyl)amino)propyl)-N′-(3-aminopropyl)propane-1,3-diamine tetrahydrochloride (156)

HCl was bubbled through a stirred solution of **147** (783mg, 0.31mmol) in CH_2CH_2 (20ml) for 15min at room temperature. Evaporation and lyophilisation gave the title compound (694mg, quant.) as a white wax: δ_H (270MHz, D_2O) 2.15 (6H, m, C*H*$_2$CH$_2$N), 3.15-3.28 (14H, m, CH_2N), 3.39 (3H, s, CH_3O), 3.65-3.76 (*ca.* 185H, CH_2O), 4.10 (1H, m, C*H*OH).

N,N-Bis(3-(N-(2-hydroxy-3-(ω-methoxyPEG 2000)propyl)amino)propyl)-N′-(3-aminopropyl)propane-1,3-diamine tetrahydrochloride (158)

HCl was bubbled through a stirred solution of **149** (1.305g, 0.28mmol) in CH_2Cl_2 (20ml) for 20min at room temperature. Evaporation and lyophilisation gave the title compound (1.096g, quant.) as a white wax: δ_H (400MHz, D_2O) 1.97-2.13 (6H, m, C*H*$_2$CH$_2$N), 3.07-3.33 (*ca.* 12H, m, CH_2N), 3.35-3.46 (*ca.* 4H, m, CH_2N), 3.37 (6H, s, CH_3O), 3.61-3.73 (*ca.* 370H, CH_2O), 4.23 (2H, m, C*H*OH).

N,N′-Bis(3-(2-cyanoethylamino)propyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (161)

To **130** (2.43g, 6.3mmol) in MeOH (20ml) was added acrylonitrile (0.86ml, 693mg, 13mmol) slowly at 0°C with stirring. After 1h the ice bath was removed and the mixture was stirred at room temperature overnight. Evaporation gave the title compound (3.03g, 98%) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.45 (18H, s, Bu^t), 1.70 (4H, qn, J 6.7Hz, C*H*$_2$CH$_2$N), 1.73-1.79 (4H, m, C*H*$_2$CH$_2$NBoc and 2× NH), 2.51 (4H, t, J 6.7Hz, CH_2CN), 2.62 (4H, t, J 6.7Hz, $CH_2CH_2CH_2N$), 2.91 (4H, t, J 6.7Hz, C*H*$_2$CH$_2$CN), 3.16-3.43 (8H, m, CH_2NBoc); *m/z* (FAB+) 496.3704 $(M+H)^+$ ($^{13}C_1C_{24}H_{47}N_6O_4$ requires 496.3692) 495.3676 $(M+H)^+$ ($C_{25}H_{47}N_6O_4$ requires 495.3659).

N,N′-Bis(3-(N-*t*-butoxycarbonyl)-N-(2-cyanoethyl)amino)propyl)-N,N′-di(*t*-butoxycarbonyl)propane-1,3-diamine (162)

To **161** (2.92g, 5.9mmol) in CH_2Cl_2 (30ml) was added di-*t*-butyl dicarbonate (2.64g, 12mmol) in CH_2Cl_2 (10ml) slowly, with stirring, at 0°C. After 90min, the ice bath was removed and the mixture was stirred at room temperature overnight. Evaporation gave the crude title compound (4.1g, quant.) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.46 (18H, s, Bu^t), 1.47 (18H, s, Bu^t), 1.69-1.88 (6H, m, C*H*$_2$CH$_2$N), 2.55-2.70 (4H, m, CH_2CN), 3.17-3.29 (12H, m, CH_2NBoc), 3.48 (4H, t, J 6.9Hz, C*H*$_2$CH$_2$CN).

This material was used immediately without further characterisation.

N,N′-Bis(3-(N-(3-aminopropyl)-N-(*t*-butoxycarbonyl)amino)propyl)-N,N′-di(-*t*-butoxycarbonyl)propane-1,3-diamine (163)

To **162** (2.580g, 3.72mmol) in MeOH (30ml) was added W-2 Raney Nickel (2g, dense slurry in water) in MeOH (10ml). The mixture was cooled to 0°C and ammonia was bubbled through for 30min. The mixture was treated with H_2 (60psi) at room temperature for 65h. The catalyst was filtered off through Celite. The catalyst and Celite pad were washed with MeOH (400ml). Evaporation of the solvent from the combined filtrate and washings, followed by chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 70:10:1, then 60:10:1, then 50:10:1) gave the title compound (2.286g, 88%) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.45 (36H, s, Bu^t), 1.66 (4H, qn, J 6.7Hz, CH_2CH_2N), 1.71-1.78 (6H, m, CH_2CH_2NBoc), 1.88 (4H, s, NH_2), 2.70 (4H, t, J 6.7Hz, C$H_2$$NH_2$), 3.17-3.43 (16H, m, CH_2NBoc); *m/z* (FAB+) 704.5381 $(M+H)^+$ ($^{13}C_1C_{34}H_{71}N_6O_8$ requires 704.5367), 703.5348 $(M+H)^+$ ($C_{35}H_{71}N_6O_8$ requires 703.5333).

N,N′-Bis(3-(3-aminopropylamino)propyl)propane-1,3-diamine hexahydrochloride (169)

HCl gas was bubbled through a stirred solution of **163** (35mg, 0.05mmol) in CH_2Cl_2 (10ml) for 20 min. Evaporation, then lyophilisation gave the title compound as a hygroscopic off-white solid (25mg, quant.): δ_H (270MHz, D_2O) 2.05-2.20 (10H, m, CH_2CH_2N), 3.08-3.26 (20H, m, CH_2N).

N′-(3-(3-Aminopropyl)-N-(*t*-butoxycarbonyl)amino)propyl-N-(3-(N-(3-(2-hydroxy-3-(ω-methoxyPEG 550)propylamino)propyl)-N-(*t*-butoxycarbonyl)amino)propyl)-N,N′-di(-*t*-butyloxycarbonyl)propane-1,3-diamine (164)

To **163** (537mg, 0.76mmol) in *iso*propanol (8ml) was added mPEG 550 oxiranylmethyl ether (465mg, 0.75mmol) in *iso*propanol (2ml). The mixture was stirred at 80-90°C for 48h. Chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 80:10:1) gave the title compound (302mg 32%) as a pale yellow oil; δ_H (400MHz; $CDCl_3$) 1.45 (36H, s, Bu^t), 1.72-1.81 (10H, m, CH_2CH_2N), 2.67-2.72 (6H, m, CH_2N), 3.14-3.30 (16H, m, CH_2NBoc), 3.38 (3H, s, CH_3O), 3.45-3.58 (*ca.* 8H, m, CH_2O) 3.62-3.70 (*ca.* 50H, m, CH_2O), 3.82 (1H, m, C*H*OH); *m/z* (FAB+) 1320 (1%), 1276 (1%), 1232 (1.5%), 1188 (1.5%), 1144 (1%) all $(M+H)^+$.

N′-(3-(3-Aminopropylamino)propyl)-N-(3-(3-(3-(2-hydroxy-3-(ω-methoxyPEG 550)propylamino)propylamino)propylamino)propyl)propane-1,3-diamine hexahydrochloride (167)

HCl was bubbled through a stirred solution of **164** (302mg, 0.33mmol) in CH_2Cl_2 (25ml) for 20min at room temperature. Evaporation gave an oil which was lyophilised to give the title compound (210mg 86%) as a white wax: δ_H (400MHz; D_2O) 1.93-2.08 (10H, m, CH_2CH_2N)

3.01-3.10 (22H, m, CH_2N), 3.24 (3H, s, CH_3O), 3.44-3.52 (*ca.* 8H, m, CH_2O), 3.52-3.60 (*ca.* 50H, m, CH_2O), 3.71-3.75 (1H, m, C*H*OH).

N,N′-Bis(3-(N-*t*-butoxycarbonyl)-N-(3-(2-hydroxy-3-(ω-methoxyPEG 550) propylamino)propyl)amino)propyl)-N,N′-di-(*t*-butyloxycarbonyl)-propane-1,3-diamine (165) and

N′-(3-(N-(*t*-butoxycarbonyl)-N-(3-(2-hydroxy-3-(ω-methoxyPEG550)propylamino) propyl)amino)propyl)-N′-(3-(N-(*t*-butoxycarbonyl)-N-(3-(N,N-bis-(2-hydroxy-3-(ω-methoxyPEG550)propyl)amino)propyl)amino)propyl)-N,N′-di-(*t*-butoxycarbonyl)propane-1,3-diamine (166)

To **163** (845mg, 1.20mmol) in *iso*propanol (25ml) was added mPEG 550 oxiranylmethyl ether (1.45g, 2.4mmol) in *iso*propanol (10ml). The mixture was stirred at 65°C for 44h. Evaporation and chromatography (CH_2Cl_2/MeOH 6:1) gave the triPEGylated conjugate(167mg, 6%) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.45 (36H, s, Bu^t), 1.73 (10H, m, C$H_2$$CH_2N$) 2.54 (10H, m, CH_2N), 3.15-3.19 (16H, m, CH_2NBoc), 3.38 (9H, s, CH_3O), 3.54-3.60 (*ca.* 20H, m, CH_2O), 3.60-3.70 (*ca.* 140H, m, CH_2O), 3.83 (3H, m, C*H*OH); *m/z* (MALDI-TOF) 2685 (99%), 2640 (99%), 2596 (100%), 2551 (100%), 2507 (100%), 2463 (100%), 2419 (99%) all $(M+H)^+$; and the diPEGylated conjugate (262mg 12%) as a colourless oil: δ_H (400MHz; $CDCl_3$) 1.45 (36H, s, Bu^t), 1.73 (10H, m, C$H_2$$CH_2N$), 2.48 (8H, m, CH_2N), 3.10-3.20 (16H, m, CH_2NBoc), 3.38 (6H, s, CH_3O), 3.45-3.60 (*ca.* 10H, m, CH_2O), 3.61-3.70 (*ca.* 100H, m, CH_2O), 3.85 (2H, m, C*H*OH).

N,N′-Bis(3-(3-(2-hydroxy-3-(ω-methoxyPEG550)propylamino) propylamino)propyl)-propane-1,3-diamine hexahydrochloride (168)

HCl was bubbled through a stirred solution of **165** (243mg, 0.13mmol) in CH_2Cl_2 (5ml) for 15min at room temperature. Evaporation gave an oil which was dissolved in water and lyophilised to give an off-white foamy solid (220mg, quant.): δ_H (400MHz; D_2O) 1.95-2.03 (10H, m, C$H_2$$CH_2N$), 3.04 (24H, t, J 7.6Hz, CH_2N), 3.22 (6H, s, CH_3O), 3.43-3.48 (*ca.* 14H, m, CH_2O), 3.53-3.63 (*ca.* 120H, m, CH_2O), 3.99 (2H, m, C*H*OH).

Bis(3-*t*-butoxycarbonylaminopropyl)amine (170)

To bis(3-aminopropyl)amine (1.40g, 10.7mmol) in dry THF (20ml) was added 2-(*t*-butoxycarbonyloxyimino)-2-phenylacetonitrile (5.78g, 23.5mmol) in dry THF (50ml) dropwise over 90min, with stirring, at 0°C. The mixture was allowed to warm to room temperature and stirred for 40min. Evaporation and chromatography (EtOAc/MeOH 4:1) gave the title compound (1.874g, 54%) as a pale yellow solid: mp 70-71°C (lit. [Hu and Hesse, 1996] mp 82°C); δ_H (270MHz, $CDCl_3$) 1.44 (18H, s, Bu^t), 1.64 (5H, qn, J 6.4Hz, CH_2CH_2 and NH), 2.65 (4H, t, J 6.4Hz, CH_2N), 3.18-3.27 (4H, m, CH_2NBoc), 5.28 (2H, s, NH); *m/z* (FAB+) 333.2580 $(M+H)^+$ ($^{13}C_1C_{15}H_{34}N_3O_4$ requires 333.2583); 332.2548 $(M+H)^+$ ($C_{16}H_{34}N_3O_4$ requires 332.2549).

N,N-Bis(3-*t*-butoxycarbonylaminopropyl)-2-cyanoethylamine (171)

To bis(3-*t*-butoxycarbonylaminopropyl)amine (1.115g, 3.39mmol) in THF (10ml) was added acrylonitrile (0.5ml, 403mg, 7mmol) and the mixture was stirred at room temperature overnight. Acrylonitrile (1ml, 806mg,14mmol) was added and the mixture was heated to reflux for 60h. Evaporation and chromatography (EtOAc) gave the title compound (1.042g, 80%) as a pale yellow oil: δ_H (270MHz, $CDCl_3$) 1.44 (18H, s, Bu^t), 1.64 (4H, qn, J 6.6Hz, CH_2CH_2N), 2.45 (2H, t, J 6.9Hz, CH_2CN), 2.48 (4H, t, J 6.7Hz, CH_2N), 2.76 (2H, t, J 7.0Hz, CH_2N), 3.15-3.22 (4H, m, CH_2NBoc), 5.12 (2H, s, NH); *m/z* (FAB+) 386.2853 $(M+H)^+$ ($^{13}C_1C_{18}H_{37}N_4O_4$ requires 386.2848); 385.2818 $(M+H)^+$ ($C_{19}H_{37}N_4O_4$ requires 385.2815).

3-Amino-N,N-bis(3-*t*-butoxycarbonylaminopropyl)propylamine (172)

To **171** (971mg, 2.54mmol) in MeOH (25ml) was added W-2 Raney nickel (1g, dense slurry in water) with MeOH (10ml). The mixture was cooled to 0°C and ammonia was bubbled through for 30min. The mixture was treated with H_2 (60psi) at room temperature for 72h. The catalyst was filtered off through Celite. The catalyst and Celite pad were washed with EtOH (300ml). Evaporation of the solvent from the combined filtrate and washings gave a blue-green oil (*ca.* 1g) which was used immediately for the next step: δ_H (400MHz, $CDCl_3$) 1.43 (20H, s, Bu^t and NH_2), 1.63-1.66 (6H, m, CH_2CH_2N), 2.44-2.75 (8H, br m, CH_2N), 3.12-3.20 (4H, br m, CH_2NBoc) 5.28 (2H, br s, NH); *m/z* (FAB+) 390.3160 $(M+H)^+$ ($^{13}C_1C_{18}H_{41}N_4O_4$ requires 390.3161); 389.3126 $(M+H)^+$ ($C_{19}H_{41}N_4O_4$ requires 389.3128).

Benzyl N-(3-(N,N-bis(3-*t*-butoxycarbonylaminopropyl)amino)propyl)carbamate (176)

To **172** (789mg, 2.04mmol) in CH_2Cl_2 (10ml) was added NEt_3 (0.6ml, 436mg). The mixture was cooled to 0°C and benzyl chloroformate (0.5ml, 2.5mmol) was added slowly in 4 portions with stirring. The mixture was stirred at 0°C for 10min then at room temperature overnight.

The mixture was diluted with CH_2Cl_2 (150ml), washed with water (100ml), then brine (100ml) and then dried with $MgSO_4$. Evaporation and chromatography (CH_2Cl_2 then CH_2Cl_2/MeOH 10:1 then 7:1) gave the title compound (301mg, 29%) as a colourless oil: δ_H (270MHz, $CDCl_3$) 1.42 (18H, s, Bu^t), 1.58-1.65 (6H, m, CH_2CH_2N), 2.41 (6H, t, J 6.5Hz, CH_2N), 3.12-3.25 (6H, m, CH_2NCO), 5.08 (2H, s, $PhCH_2$), 5.29 (2H, m, NH), 5.90 (1H, br s, NH), 7.30-7.34 (5H, m, Ar); *m/z* (FAB+) 524.3529 $(M+H)^+$ ($^{13}C_1C_{26}H_{47}N_4O_6$ requires 524.3529); 523.3497 $(M+H)^+$ ($C_{27}H_{47}N_4O_6$ requires 523.3496).

Benzyl N-(3-(N,N-bis(3-aminopropyl)amino)propyl)carbamate trihydrochloride (177)

HCl was bubbled through a solution of **176** (245mg, 0.47mmol) in CH_2Cl_2 (15ml) at room temperature for 30min. Evaporation then lyophilisation gave the title compound (191mg, 98%) as a colourless glass: δ_H (270MHz, D_2O) 1.90-2.00 (2H, m, CH_2CH_2N), 2.00-2.21 (4H, m, CH_2CH_2N), 3.02-3.15 (4H, m, CH_2N), 3.19-3.45 (8H, m, CH_2N), 5.12 (2H, s, $PhCH_2$), 7.41-7.45 (5H, m, Ar); *m/z* (FAB+) 324.2486 $(M+H)^+$ ($^{13}C_1C_{16}H_{31}N_4O_2$ requires 324.2481); 323.2454 $(M+H)^+$ ($C_{17}H_{31}N_4O_2$ requires 323.2447).

Benzyl N-(3-(N,N-bis(3-(N,N-bis(2-cyanoethyl)amino)propyl)amino)propyl) carbamate (179)

To **177** (181mg, 0.56mmol) in MeOH (10ml) was added NEt_3 (0.24ml, 174mg, 1.7mmol) then acrylonitrile (0.06ml, 48mg, 0.9mmol). The mixture was stirred at room temperature overnight. Acrylonitrile (0.06ml, 48mg, 0.9mmol) was added and the mixture was stirred at room temperature for 48h. Evaporation and chromatography (CH_2Cl_2/MeOH 7:1) gave the title compound (80mg, 26%) as a colourless oil: δ_H (270MHz, $CDCl_3$) 1.68 (6H, qn, J 7.0Hz, CH_2CH_2N), 2.44 (8H, t, J 6.6Hz, CH_2), 2.55 (10H, t, J 6.8Hz, CH_2), 2.79 (8H, t, J 6.5Hz, CH_2), 3.23-3.30 (2H, m, CH_2NCO), 5.09 (2H, s, $PhCH_2$), 5.81 (1H, m, NH), 7.30-7.37 (5H, m, Ar); *m/z* (FAB+) 557 [$(M+Na)^+$, 10%], 535 [$(M+H)^+$, 100%] 91 [$(C_7H_7)^+$, 40%].

ω-Methoxy PEG 550 chloroformate

To mPEG 550 (10.0g, 0.018mol, previously dried by azeotropic removal of water) in CH_2Cl_2 (150ml) was added phosgene (*CAUTION! highly toxic*, 100ml, 20% solution in toluene). The mixture was stirred at room temperature for 48h and then stored in solution in the CH_2Cl_2/toluene/phosgene mixture. An aliquot was removed when required and the solvents evaporated to yield a colourless oil: δ_H (270MHz, $CDCl_3$) 3.38 (3H, s, CH_3O), 3.54 (2H, m, CH_2O), 3.60-3.70 (*ca.* 45H, m, CH_2O), 3.78 (2H, m, CH_2O), 4.46 (2H, m, CH_2OCO); *m/z* (FAB+) 777 (8%), 733 (10%), 689 (12%), 645 (14%), 601 (14%), 557 (12%), 513 (8%) all $(M+Na)^+$, 755 (7%), 711 (8%), 667 (10%), 623 (12%), 579 (13%), 535 (12%) all $(M+H)^+$.

ω-MethoxyPEG550yl N-(3-(N,N-bis(3-*t*-butoxycarbonylaminopropyl)amino) propyl)carbamate (174)

To **172** (94mg, 0.24mmol) in CH_2Cl_2 (5ml) was added NEt_3 (0.07ml, 51mg, 0.5mmol) followed by mPEG 550 chloroformate (192mg, 0.3mmol). The mixture was stirred at room temperature overnight. Evaporation and chromatography (CH_2Cl_2/MeOH 7:1) gave the title compound (65mg, 28%) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.44 (18H, s, Bu^t), 1.63-1.66 (6H, m, C*H*$_2$CH$_2$N), 2.44-2.50 (6H, m, CH_2N), 3.12-3.25 (6H, m, CH_2NCO), 3.38 (3H, s, CH_3O), 3.54-3.56 (2H, m, CH_2O), 3.62-3.70 (*ca.* 40H, m, CH_2O), 4.19-4.21 (2H, m, CH_2OCON), 5.27 (2H, br s, HNBoc), 5.64 (1H, br s, NH); *m/z* (FAB+) 1019 (25%), 975 (40%), 931 (46%), 887 (55%), 843 (57%), 799 (50%), 755 (44%), 711 (30%) all $(M+H)^+$.

ω-MethoxyPEG550yl N-(3-(N,N-bis(3-*t*-butoxycarbonylaminopropyl) amino)propyl)carbamate trihydrochloride (175)

HCl was bubbled through a solution of **174** (38mg, 0.04mmol) in CH_2Cl_2 (3ml) for 15min. Evaporation and lyophilisation gave the title compound (29mg, quant.) as a colourless oil: δ_H (400MHz, D_2O) 1.75-1.81 (2H, m, C*H*$_2$CH$_2$N), 1.92-2.02 (4 H, m, C*H*$_2$CH$_2$N), 2.94 (4H, t, J 7.7Hz, CH_2N), 3.07-3.16 (8H, m, CH_2N), 3.22 (3H, s, CH_3O), 3.46-3.48 (2H, m, CH_2O), 3.54-3.61 (*ca.* 40H, m, CH_2O), 4.06 (2H, m, CH_2CON); *m/z* (FAB+) 863 (40%), 819 (54%), 775 (66%), 732 (81%), 687 (84%), 643 (86%), 599 (72%), 555 (52%), 511 (35%) all $(M+H)^+$.

Bis(3-trifluoroacetamidopropyl)amine (182)

To a solution of bis(3-aminopropyl)amine (15.0g, 0.115mol) in absolute EtOH (100ml) was added ethyl trifluoroacetate (33.4g, 0.235mol) slowly, with stirring, at 0°C. After 20min the mixture was allowed to reach room temperature and stirred for a further 60h. The solvents were evaporated and the residue dissolved in EtOAc (500ml) and washed with water (200ml), then brine (200ml), then dried with $MgSO_4$. Evaporation gave the title compound (37g, quant.) as a pale yellow solid: mp 168-171°C; δ_H (270MHz, $CDCl_3$) 1.74 (4H, qn, J 6.3Hz, C*H*$_2$CH$_2$N), 2.73 (4H, t, J 6.3Hz, CH_2N), 3.44 (4H, t, J 6.3Hz, CH_2NCO), 8.67 (2H, br s, NH); δ_C (68MHz, $CDCl_3$) 27.85 (*C*H$_2$CH$_2$N), 39.26 (CH_2N), 47.87 (*C*H$_2$NCO), 116.11 (q, J 288Hz, CF_3), 157.30 (q, J 37Hz, CO); *m/z* (FAB+) 325.1192 $(M+H)^+$ ($^{13}C_1C_9H_{16}F_6N_3O_2$ requires 325.1180); 324.1162 $(M+H)^+$ ($C_{10}H_{16}F_6N_3O_2$ requires 324.1146).

Ethyl 6-bromohexanoate (184)

To 6-bromohexanoic acid (10.0g, 0.05mol) in ethanol (500ml) was added concentrated H_2SO_4 (4ml). The mixture was heated to reflux for 24h. The solvents were evaporated to low volume (*ca.* 70ml) then diluted with CH_2Cl_2 (200ml). The solution was washed with water (200ml) and then the water layer was extracted with CH_2Cl_2 (100ml). The pooled organic phases were

washed with 8% aqueous $NaHCO_3$ solution (200ml), then with brine (200ml), then dried with $MgSO_4$. Evaporation followed by kugelrohr distillation gave the title compound (9.95g, 87%) as a colourless liquid: $bp_{0.08}$ 125°C (lit. [McHintosh *et al*, 1983] $bp_{0.13}$ 69-72°C); δ_H (400MHz, $CDCl_3$) 1.25 (3H, t, J 7.0Hz, CH_3), 1.48 (2H, qn, J 7.0Hz, CH_2), 1.66 (2H, qn, J 7.0Hz, CH_2), 1.89 (2H, qn, J 7.0Hz, CH_2), 2.32 (2H, t, J 7.0Hz, CH_2CO), 3.41 (2H, t, J 7.0Hz, CH_2Br), 4.12 (2H, q, J 7.0Hz, CH_2CH_3).

Ethyl 6-(bis(3-trifluoroacetamidopropyl)amino)hexanoate (185)

To bis(3-trifluoroacetamidopropyl)amine (5.00g, 15mmol) in DMF (10ml) was added anhydrous K_2CO_3 (2.5g, 18mmol) and ethyl 6-bromohexanoate (3.79g,17mmol). The mixture was stirred at 80°C overnight. The solvents were evaporated and the residue taken up in water and extracted with CH_2Cl_2 (3× 100ml). The pooled organic phases were washed with brine (200ml) then dried with $MgSO_4$. Evaporation and chromatography (EtOAc/hexane 10:1) gave the title compound (3.03g, 42%) as a colourless oil: δ_H (270MHz, $CDCl_3$) 1.23-1.33 (5H, m, CH_3 and $CH_2CH_2CH_2N$), 1.41-1.52 (2H, m, CH_2CH_2CO), 1.58-1.79 (6H, m, CH_2CH_2N), 2.30 (2H, t, J 7.3Hz, CH_2CO), 2.43 (2H, t, J 7.1Hz, CH_2N), 2.54 (4H, t, J 6.4Hz, CH_2N), 3.40-3.49 (4H, m, CH_2NCO), 4.12 (2H, q, J 7.2Hz, CH_2O), 8.54 (2H, s, NH); *m/z* (FAB–) 464 [$(M-H)^-$, 100%]; (FAB+) 488 [$(M+Na)^+$, 20%], 467.2175 $(M+H)^+$ ($^{13}C_1C_{17}H_{30}F_6N_3O_4$ requires 467.2174); 466.2141 $(M+H)^+$ ($C_{18}H_{30}F_6N_3O_4$ requires 466.2141).

***t*-Butyl N-(2-aminoethyl)carbamate (193)**

To ethane-1,2-diamine (3.1ml, 46mmol) in THF (20ml) was added di-*t*-butyl dicarbonate (500mg, 2.3mmol) in THF (20ml) dropwise over 15min, with stirring, at room temperature. The mixture was stirred at room temperature for a further 3h, then the solvents were evaporated. The residue was dissolved in chloroform/MeOH (200ml/3ml) and washed with water (2× 200ml) then brine (200ml), then dried with $MgSO_4$. Evaporation gave the title compound (210mg, 57%) as a colourless oil: δ_H (400MHz $CDCl_3$) 1.45 (11H, m, Bu^t and NH_2), 2.80 (2H, t, J 5.8Hz, CH_2N), 3.15-3.18 (2H, m, CH_2NBoc), 4.95 (1H, s, NH); *m/z* (FAB+) 161 [(M+H)+, 95%].

6-Bromo-N-(2-(*t*-butoxycarbonylamino)ethyl)hexanamide (195)

To 6-bromohexanoic acid (1.535g, 7.87mmol) in dry CH_2Cl_2 (15ml) was added oxalyl chloride (5.0g, 39mmol). The mixture was cooled to 0°C and DMF (2 drops) was added (*CAUTION! rapid evolution of gas*). The mixture was stirred at 0°C for 30min then at room temperature for 100min. The solvents were evaporated and dry CH_2Cl_2 (5ml) was added then evaporated. Dry CH_2Cl_2 (20ml) was added, then **193** (2.04g, 12.5mmol) and NEt_3 (2.5ml, 1.82g) slowly at 0°C. The mixture was stirred at 0°C for 30min then at room temperature for 90min. The mixture

was diluted with CH_2Cl_2 (100ml) then washed with water (100ml) then brine (100ml) then dried with $MgSO_4$. Evaporation then recrystallisation (EtOAc) gave the title compound (1.795g, 68%) as white crystals: mp 104-106°C; δ_H (270MHz, $CDCl_3$) 1.44 (9H, s, Bu^t), 1.44-1.51 (2H, m, CH_2CH$_2$CH$_2$CO), 1.66 (2H, qn, J 7.2Hz, CH_2CH$_2$CO), 1.87 (2H, qn, J 7.2Hz, CH_2CH$_2$Br), 2.20 (2H, t, J 7.3Hz, COCH$_2$), 3.27-3.37 (4H, m, CH$_2$NCO), 3.41 (2H, t, J 6.8Hz, CH$_2$Br), 5.04 (1H, s, NH), 6.38 (1H, s, NH); *m/z* (FAB+) 340.1118 $(M+H)^+$ ($^{13}C_1C_{12}H_{26}{}^{81}BrN_2O_3$ requires 340.1139); 339.1097 $(M+H)^+$ ($C_{13}H_{26}{}^{81}BrN_2O_3$ requires 339.1106); 338.1113 $(M+H)^+$ ($^{13}C_1C_{12}H_{26}{}^{79}BrN_2O_3$ requires 338.1160); 337.1121 $(M+H)^+$ ($C_{13}H_{26}{}^{79}BrN_2O_3$ requires 337.1127).

6-(Bis(3-trifluoroacetamidopropyl)amino)-N-(2-*t*-butoxycarbonylaminoethyl) hexanamide (196)

Compound **195** (557mg, 1.65mmol), bis(3-trifluoroacetamidopropyl)amine (587mg, 1.82mmol) and anhydrous K_2CO_3 (340mg, 2.5mmol) in dry DMF (3ml) were heated to 80°C with stirring. After 18h, the solvents were evaporated and the was residue taken up in CH_2Cl_2 (150ml), then washed with water (100ml), then brine (100ml) and then dried with $MgSO_4$. Evaporation and chromatography (EtOAc/MeOH 10:1, then 7:1, then 5:1) gave the title compound (647mg, 68%) as a colourless oil: δ_H (270MHz, $CDCl_3$) 1.28-1.33 (2H, m, CH_2CH$_2$CH$_2$CO), 1.44 (11H, m, Bu^t and CH_2CH$_2$CO), 1.61-1.75 (6H, m, CH_2CH$_2$N), 2.18 (2H, t, J 7.3Hz, CH$_2$CO), 2.41 (2H, t, J 7.0Hz, CH$_2$N), 2.51 (4H, t, J 6.6Hz, NCH_2CH$_2$CH$_2$COCF$_3$), 3.27-3.43 (8H, m, CH$_2$NCO), 5.06 (1H, s, NH), 6.45 (1H, s, NH), 8.27 (2H, s, NHCOCF$_3$); *m/z* (FAB+) 602 [$(M+Na)^+$, 20%], 581.2970 $(M+H)^+$ ($^{13}C_1C_{22}H_{40}F_6N_5O_5$ requires 581.2967), 580.2940 $(M+H)^+$ ($C_{23}H_{40}F_6N_5O_5$ requires 580.2934), (FAB–) 578 [$(M-H)^-$, 100%].

***t*-Butyl N,N′-bis(3-trifluoroacetamidopropyl)carbamate (200)**

To bis(3-trifluoroacetamidopropyl)amine (270mg, 0.84mmol) in CH_2Cl_2 (5ml) was added di-*t*-butyl dicarbonate (182mg, 0.84mmol) in CH_2Cl_2 (5ml) slowly, with stirring, at 0°C. The mixture was stirred at 0°C for 10min, then at room temperature for 48h. Evaporation gave the title compound (360mg, quant.) as a colourless oil: δ_H (400MHz, $CDCl_3$, 23°C) 1.47 (9H, s, Bu^t), 1.68-1.86 (4H, m, CH_2CH$_2$N), 3.25-3.20 (8H, m, CH$_2$NCO), 6.90 (1H, br s, NH), 8.24 (1H, br s, NH); δ_H (400MHz, $CDCl_3$, –40°C) 1.47 (9H, s, Bu^t), 1.58-1.63 (2H, m, CH_2CH$_2$N), 1.83 (2H, qn, J 7.4Hz, CH_2CH$_2$N), 3.22 (2H, t, J 7.0Hz, CH$_2$NBoc), 3.28-3.35 (4H, m, CH$_2$NCO), 3.37 (2H, dt, J 5.4, 7.0Hz, CH$_2$NHCOCF$_3$), 7.52-7.59 (1H, m, NH), 8.69 (1H, t, J 5.8Hz, NH); *m/z* (FAB+) 446 [$(M+Na)^+$, 15%], 424 [$(M+H)^+$, 20%]; (FAB–) 422 [$(M-H)^-$, 100%].

t-Butyl N,N-bis(3-aminopropyl)carbamate (201)

To **200** (310mg, 0.73mmol) in MeOH (6ml) was added concentrated aqueous ammonia solution (3ml). Ammonia was bubbled through the solution for 5min at 0°C, then the mixture was heated at 60°C in a sealed bottle for 5h. Evaporation and chromatography (CH_2Cl_2/MeOH/conc. aqueous NH_3 10:5:1) gave the title compound (155mg, 91%) as a colourless oil: δ_H (400MHz, $[^2H]_6$-DMSO) 1.41 (9H, s, Bu^t), 1.75 (4H, qn, J 7.0Hz, CH_2CH_2N), 2.76 (4H, t, J 7.3Hz, CH_2N), 3.18 (4H, t, J 6.8Hz, CH_2NBoc), 3.38 (4H, br s, NH_2); *m/z* (FAB+) 254 [$(M+Na)^+$, 8%], 232 [$(M+H)^+$, 100%], 132 [$(M-Boc+H)^+$, 64%].

t-Butyl N,N-bis(3-(2-cyanoethylamino)propyl)carbamate (202)

To bis(3-trifluoroacetamidopropyl)amine (3.36g, 10.4mmol) in THF (50ml) was added di-*t*-butyl dicarbonate (2.27g, 10.4mmol) in THF (50ml) slowly over 15min at room temperature. The mixture was stirred at room temperature for 24h, then the solvents were evaporated. To the residue in MeOH (100ml) was added concentrated aqueous ammonia solution (50ml), then ammonia was bubbled through the solution for 30min at 0°C. The mixture was stirred at room temperature in a sealed bottle for 72h. Evaporation and chromatography (CH_2Cl_2/ MeOH/conc. aqueous NH_3 10:5:1) gave a colourless oil (*ca.* 2.4g) which was dissolved in MeOH (50ml). Acrylonitrile (1.5ml, 1.21g, 23mmol) in MeOH (10ml) was added slowly at room temperature. The mixture was stirred at room temperature for 40h. The solvents were evaporated and the residue was dissolved in CH_2Cl_2 (200ml) and washed with water (100ml), then brine (100ml), then dried with $MgSO_4$. Evaporation gave a colourless oil (800mg), fraction A.

Evaporation of the water from the aqueous layer gave an oily residue (*ca.* 2g), to this, in MeOH (30ml), was added acrylonitrile (0.6ml, 484mg, 9.1mmol) in MeOH (10ml). The mixture was stirred at room temperature for 40h then acrylonitrile (1.2ml, 967mg, 18.2mmol) was added and stirring was continued for a further 48h. Anhydrous K_2CO_3 (1.5g, 10.9mmol) was added and the mixture was stirred at room temperature for 14h. The solvents were evaporated and the residue was taken up in CH_2Cl_2 (200ml), washed with water (150ml), then brine (150ml) and then dried with $MgSO_4$. Evaporation gave a pale brown oil (ca 1.2g) fraction B.

Fraction A and fraction B were combined. Chromatography (CH_2Cl_2/MeOH 4:1 then 3:1) gave the title compound (1.04g, 30%) as a pale yellow oil: δ_H (270MHz, $CDCl_3$) 1.46 (9H, s, Bu^t),1.71-1.73 (6H, m, CH_2CH_2N and NH), 2.50 (4H, t, J 6.5Hz, CH_2CN), 2.63 (4H, t, J 6.6Hz, NCH_2CH_2CN), 2.89 (4H, t, J 6.6Hz, CH_2N), 3.20-3.32 (4H, m, CH_2NBoc); *m/z* (FAB+) 339.2588 $(M+H)^+$ ($^{13}C_1C_{16}H_{32}N_5O_2$ requires 339.2589); 338.2566 $(M+H)^+$ ($C_{17}H_{32}N_5O_2$ requires 338.2556); 238 [$(M-Boc+H)^+$ 56%].

t-Butyl N,N-bis(3-(3-aminopropylamino)propyl)carbamate (203)

To **202** (1.00g, 3.0mmol) in MeOH (20ml) was added W-2 Raney nickel (1g, dense slurry in water). Ammonia was bubbled through the mixture for 30min at 0°C. The mixture was treated with H_2 (60psi) at room temperature for 60h. The catalyst was filtered off through Celite. The Celite pad and catalyst were washed with MeOH (300ml). Evaporation of the combined filtrate and washings gave a blue oil (*ca.* 1g) which was used in the next step without purification.

t-Butyl N,N-bis(3-(N-benzyloxycarbonyl-N-(3-benzyloxycarbonylaminopropyl) aminopropyl))carbamate (204)

To **203** (*ca.* 1g) in THF (20ml) was added dibenzyl dicarbonate (4.23g, 15mmol) in THF (10ml) in 10 portions, with stirring, at 0°C. The mixture was stirred at 0°C for 20min then at room temperature for 36h. Evaporation and chromatography (EtOAc/hexane 1:1, then 2:1) gave the title compound (1.57g, ~60%) as a colourless oil: δ_H (270MHz, $CDCl_3$) 1.40 (9H, s, Bu^t), 1.61-1.78 (8H, m, CH_2CH_2N), 3.05-3.38 (16H, m, CH_2NCO), 5.09 (8H, s, $PhCH_2$), 5.68 (2H, br s, NH), 7.28-7.36 (20H, m, Ar); *m/z* (FAB+) 904 [$(M+Na)^+$, 16%], 883.4661 $(M+H)^+$ ($^{13}C_1C_{48}H_{64}N_5O_{10}$ requires 883.4687); 882.4646 $(M+H)^+$ ($C_{49}H_{64}N_5O_{10}$ requires 882.4653), 782 [$(M-Boc+H)^+$, 100%].

Bis(3-(N-benzyloxycarbonyl-N-(3-benzyloxycarbonyl)aminopropyl) aminopropyl)amine (205)

HCl was bubbled through a solution of **204** (1.327g, 1.51mmol) in CH_2Cl_2 (40ml) for 30min at room temperature. The solvents were evaporated, the residue was dissolved in MeOH (10ml) and aqueous 5M NaOH (0.9ml) was added. Evaporation gave an oily residue which was taken up in CH_2Cl_2 (200ml), washed with water (100ml), then brine (100ml) and then dried with $MgSO_4$. Evaporation gave the title compound (1.074g, 91%) as a colourless oil: δ_H (270MHz, $CDCl_3$) 1.55-1.77 (8H, m, CH_2CH_2N), 2.10 (1H, br s, NH), 2.40-2.58 (4H, m, CH_2N), 3.06-3.39 (12H, m, CH_2NCbz), 5.10 (8H, s, $PhCH_2$), 5.69 (2H, br s, NH), 7.30-7.35 (20H, m, Ar); *m/z* (FAB+) 783.4168 $(M+H)^+$ ($^{13}C_1C_{43}H_{56}N_5O_8$ requires 783.4162); 782.4139 $(M+H)^+$ ($C_{44}H_{56}N_5O_8$ requires 782.4129).

Bis(3-(N-benzyloxycarbonyl-N-(3-benzyloxycarbonyl)aminopropyl)aminopropyl) amine-N-(2-(t-butoxycarbonylamino)ethyl)hexanamide (206)

To the amine **205** (1.074g, 1.38mmol) in dry DMF (4ml) was added anhydrous K_2CO_3 (285mg, 2mmol) and **195** (693mg, 2.1mmol). The mixture was stirred at 85°C for 20h, then the solvents were evaporated. The residue was taken up in CH_2Cl_2 (200ml), then washed with water(100ml), then brine (100ml) and then dried with $MgSO_4$. Evaporation then chromatography (EtOAc/MeOH 6:1, then 4:1) gave the title compound (965mg, 67%) as a colourless oil: δ_H

(400MHz, $CDCl_3$) 1.24-1.42 (4H, m, $CH_2CH_2CH_2CO$), 1.42 (9H, s, Bu^t), 1.59-1.68 (10H, m, CH_2CH_2N), 2.12-2.16 (2H, m, CH_2CO), 2.25-2.40 (6H, m, CH_2N), 3.15-3.29 (16H, m, CH_2NCO), 5.10 (8H, s, $PhCH_2$), 5.74 (1H, m, NH), 6.43 (1H, m, NH), 7.31-7.33 (20H, m, Ar); δ_C (100MHz, $CDCl_3$) 25.24, 25.57, 25.84, 26.10, 26.44, 26.98, 28.10 (CH_2), 28.37 (CH_3), 28.91, 36.41, 37.69, 38.28, 40.38, 44.38, 45.24, 46.01, 51.17, 51.28, 53.44 (CH_2), 66.50, 67.15 ($PhCH_2$), 79.52 (Boc C), 127.80, 128.05, 128.31, 128.47 (Ar CH), 136.01, 136.50, 136.74 (Ar C), 156.02, 156.58, 156.91 (CO, *carbamates*), 173.74 (CO, *amide*); *m/z* (FAB+) 1040.5960 $(M+H)^+$ ($^{13}C_2C_{55}H_{80}N_7O_{11}$ requires 1040.5983); 1039.5912 $(M+H)^+$ ($^{13}C_1C_{56}H_{80}N_7O_{11}$ requires 1039.5949); 1038.5893 $(M+H)^+$ ($C_{57}H_{80}N_7O_{11}$ requires 1038.5915).

Bis(3-(N-benzyloxycarbonyl-N-(3-benzyloxycarbonyl)aminopropyl)aminopropyl) amine-N-(2-aminoethyl)hexanamide (207)

HCl was bubbled through a solution of **206** (860mg, 0.83mmol) in CH_2Cl_2 (25ml) for 30min at room temperature. The solvents were evaporated, the residue was dissolved in MeOH (20ml) and aqueous 5M NaOH (0.66ml) was added. Evaporation gave an oily residue which was taken up in CH_2Cl_2 (200ml), washed with water (100ml), then brine (100ml) and then dried with $MgSO_4$. Evaporation gave the title compound (676mg, 87%) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.25-1.33 (4H, m, $CH_2CH_2CH_2CO$), 1.61-1.68 (10H, m, CH_2CH_2N), 2.03-2.16 (4H, m, CH_2NH_2 and CH_2CO), 2.25-2.35 (6H, m, CH_2N), 2.78 (2H, s, NH_2), 3.15-3.26 (14H, m, CH_2NCO), 5.10 (8H, s, $PhCH_2$), 5.75-5.77 (1H, m, NH), 6.26-6.29 (1H, m, NH), 7.31-7.33 (20H, m, Ar); *m/z* (FAB+) 939.5412 $(M+H)^+$ ($^{13}C_1C_{51}H_{72}N_7O_9$ requires 939.5425); 938.5383 $(M+H)^+$ ($C_{52}H_{72}N_7O_9$ requires 938.5391).

ω-MethoxyPEG550yl N-(2-(6-(N,N-bis(3-(N-benzyloxycarbonyl-N-(3-benzyloxycarbonylaminopropyl)amino)propyl)amino)hexanamido)ethyl) carbamate (208)

To **207** (408mg, 0.44mmol) in CH_2Cl_2 (6ml) was added mPEG 550 chloroformate (320mg, 0.5mmol) and NEt_3 (145µl, 105mg) at 0°C. The mixture was stirred at 0°C for 5min then at room temperature for 3h. Further mPEG 550 chloroformate (45mg, 0.07mmol) in CH_2Cl_2 (1ml) was added and the mixture was stirred at room temperature for 4h. Evaporation and chromatography (CH_2Cl_2/MeOH 20: 1 then 10:1) gave the title compound (274mg, 42%) as a colourless oil: δ_H (400MHz, $CDCl_3$) 1.25-1.38 (4H, m, $CH_2CH_2CH_2CO$), 1.60-1.70 (10H, m, CH_2CH_2N), 2.13-2.16 (2H, m, CH_2CO), 2.24-2.38 (6H, m, CH_2N), 3.15-3.31 (16H, m, CH_2NCO), 3.38 (3H, s, OCH_3), 3.53-3.56 (2H, m, CH_2O), 3.62-3.82 (*ca.* 40H, m, CH_2O), 4.17-4.20 (2H, m, CH_2OCO), 5.10 (8H, s, $PhCH_2$), 5.51-5.53 (1H, m, NH), 5.71 (1H, m, NH), 7.32-7.34 (20H, m, Ar); *m/z* (FAB+) $^{13}C/^{12}C$ peak cluster centred at 1481 (3%), 1438 (4%), 1393 (5%), 1349 (6%), 1305 (6%), 1261 (5%), 1217 (4%) all $(M+H)^+$.

ω-MethoxyPEG550yl N-(2-(6-(N,N-bis(3-(3-aminopropylamino)propyl) amino)hexanamido)ethyl)carbamate pentahydrochloride (209)

To **208** (75mg) in MeOH (5ml) was added Pearlman's catalyst (50mg). The mixture was treated with H_2 (60psi) at room temperature for 40h. The mixture was diluted with MeOH (10ml) and the catalyst was filtered off. Aqueous 5M HCl (2ml) was added then evaporated. Lyophilisation gave the title compound (57mg, quant.) as a colourless foam: δ_H (400MHz, D_2O) 1.37 (2H, qn, *J* 7.0Hz, C*H*$_2$CH$_2$CH$_2$CO), 1.60-1.71 (4H, m, CH$_2$), 2.06-2.15 (8H, m, C*H*$_2$CH$_2$N), 2.27 (2H, t, *J* 7.0Hz, CH$_2$CO), 3.09-3.30 (22H, m, CH$_2$NCO and CH$_2$N), 3.38 (3H, s, CH$_3$O), 3.62-3.66 (2H, m, CH$_2$O), 3.70-3.75 (*ca.* 40H, m, CH$_2$O), 4.21-4.23 (2H, m, CH$_2$OCO); *m/z* (FAB+) 944 (15%), 900 (22%), 856 (32%), 812 (35%), 768 (30%), 724 (30%), 680 (22%) all $(M+H)^+$.

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